

INVESTIGATING THE ANTIDIABETIC AND ANTIOXIDATIVE POTENTIAL OF PEELS FROM SELECTED SOUTH AFRICAN FRUITS

Gloria Otito Izu

Dissertation submitted in fulfilment of the academic requirement for the degree

Doctor of Health Sciences in Biomedical Technology

In the

Department of Health Sciences

Central University of Technology, Free State

October 2024

Supervisor: Dr Chika Ifeanyi Chukwuma (PhD: Biochemistry)

Co-supervisor: Prof. Samson S. Mashele (PhD: Medicinal Chemistry)

DECLARATION OF INDEPENDENT WORK

I Gloria Otito Izu, student number #####, hereby declare that this research project submitted to the Central University of Technology, Free State for the degree DOCTOR OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY is my independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification. As such, where external sources were utilized, due acknowledgment was given by means of a comprehensive list of references in accordance with departmental requirements. I therefore give copyright of this dissertation in favour of the Central University of Technology.

Candidate: Gloria Otito Izu

27 September 2024

Date

Supervisor: Dr Chika I. Chukwuma

30 - 09 - 2024

Date

Co-supervisor: Prof. Samson S. Mashele

30/09/2024

Date

ACKNOWLEDGEMENTS

First, I would love to express my sincere appreciation to my Principal Supervisor, Dr Chukwuma Chika, for his advice, support, motivation, patience, and immense knowledge without which I wouldn't have completed this work in record time. Dr Chukwuma Chika's attention to detail and constant feedback sharpened my writing skills and improved my thinking. I also want to thank my co-supervisor, Prof Samson Sitheni Mashele, for his critical professional insight, selfless time, and support that assisted me greatly in making a success out of this research.

My gratitude also goes to Dr. Susanna L. Bonnet, Dr. Anwar E.M. Noreljaleel, and Prof. Mamello P. Sekhoacha for their tremendous research support during my PhD program. I would like to thank my senior colleagues (Dr. Gaetan T. Tabakam, Dr. Emmanuel Mfotie Njoya, Dr. Yves M. Nguekeu Mba) and peers (Jennifer Nambooze and Maria S. Chukwuma) for their technical support.

I would like to express my appreciation to the National Research Foundation for awarding me the NRF -TWAS Doctoral Scholarship to undertake this doctoral study. I am grateful to the Central University of Technology and the University of the Free State for financial support and for providing an environment conducive to undertake this study.

I want to appreciate my parents, Mr and Mrs Pius and Celina Izu who made tremendous sacrifices for the education of their children. I am grateful for your support, prayer, and encouragement that have taken me this far. To my dear brother and his wife, Mr. and Mrs. Chuks and Rhoda Izu, I would like to express my gratitude for your tremendous financial and emotional support in ensuring my success in obtaining a Doctoral Degree.

I am also indebted to my life mentors; Dr. Olugbeko Smart, Olatunji Abiodun (SAN), Dr Ibrahim Abdullahi, Dr Adeyi Oluwatosin, and Mr. Adetola Morenikeji for their constructive advice and support.

I am blessed with the following friends, who are religiously committed to my success and well-being: Prof Oke, Dr. Iyabo Alaga, Dr. Eniola Anifalaje, Dr. Senbore Samson, Dr. Daniel Oladele, Dr Sodiq Alimi and Late Maureen Efenovwe. I also thank my pastor and his wife (Pastor and Mrs Felix Adetomi) and the entire pastorate and members of LFC, Bloemfontein for their spiritual support and love.

Finally, I give all the glory and adoration to the Almighty God who made it possible for me to complete this program with resounding success. Though, it tarries but the victory is sweet. My God, thank you for letting me through all the difficulties, disappointments, persecutions, and betrayals I experienced in the course of this program. I appreciate your divine mercy; I will keep trusting you for my future. Thank you, my Lord.

ABSTRACT

Background

There are many complications of diabetes. Oxidative stress has been reported as a major underlying mediator. Medicinal plants, including fruits and vegetables are known to contain dietary antioxidant phytoconstituents, which influences their pharmacological potentials, including mitigating the progression of degenerative diseases, such as diabetes and associated complications. Fruits like avocado (*Persea americana* Mill) and litchi (*Litchi chinensis* Sonn.) are widely cultivated for their commercial value, nutritional value and health benefits. The antioxidant and glycaemic control potentials of these fruits have been documented, which has been linked to some bioactive phenolic and flavonoid compounds. Despite being medicinally underutilized, the wastes, including the peel from these fruits have also been shown to contain these bioactive phenolic and flavonoid compounds, which are key in potentiating the documented oxidative stress and diabetes related pharmacological potentials of the peel from these fruits. However, antioxidant and glycaemic control assay-guided assay studies to better understand the antioxidant and glycaemic control phytochemistry of the peel from these fruits are scarce, which discourages their effective therapeutic applications. Therefore, this study was undertaken to investigate the antidiabetic and antioxidative phytochemistry of the peel of avocado and litchi fruit using a bioassay-guided approach

Methodology

Litchi (McLean's Red cultivar) and avocado (Var. Fuerte) fruits were purchased from a local fruit and vegetable store in Bloemfontein, South Africa. The peel of the fruits was dried, pulverized, defatted using hexane and sequentially extracted with dichloromethane, ethyl acetate, methanol, and water, in order of increasing polarity. *In vitro*, phytochemical models, namely antioxidant (Fe^{3+} reducing, radical scavenging, and anti-linoleic acid peroxidative) and glycaemic control (α -glucosidase and α -amylase inhibitory and glucose uptake modulatory), were employed for the bioassay-guided isolation, while the potent fractions or isolated compound(s) was characterized using mass spectrometry and mass spectrometry and assessed for dose-dependent inhibition of α -glucosidase and lipopolysaccharide (LPS)-induced cellular ROS production, as well as modulation of cellular glucose uptake. The radical scavenging mechanism of principal compound(s) was investigated using Density Functional Theory (DFT). Also, the ameliorative potential and possible underlying mechanisms of litchi peel ethyl acetate extract on type 2 diabetes related pathologies in a fructose/streptozotocin (STZ) model of diabetic rats was investigated.

Results and discussion

The methanol extract of avocado peel had the highest phenol content and radical scavenging activity. Fractionation of this extract yield 9 fractions (fractions 2 to 10). Fraction 8 (eluted with hexane:chloroform:methanol volume ratio of 3:6.5:0.5, respectively) had high phenolic content, demonstrated the most potent radical scavenging and showed the predominant presence of Chlorogenic acid (103.5 mg/mL) and 1-*O*-caffeoylquinic acid (102.3 mg/mL). Fraction 8 and chlorogenic acid dose-dependently inhibited *in vitro* ($IC_{50} = 5.73$ and $6.17 \mu\text{g/mL}$) and cellular ($IC_{50} = 15.9$ and $9.34 \mu\text{g/mL}$) FeSO_4 -induced lipid peroxidation, as well as LPS-induced ROS ($IC_{50} = 39.6$ and $28.2 \mu\text{g/mL}$) and NO ($IC_{50} = 63.5$ and $107 \mu\text{g/mL}$) production, while modulating antioxidant enzyme activity. DFT analysis suggest that an electron transfer, followed by proton transfer at carbons 3'OH and 4'OH positions may be the radical scavenging mechanism of chlorogenic acid. For litchi peel extracts, the ethyl acetate extract had the highest flavonoid contents, which perhaps influenced its potent anti-lipid peroxidative (65.0%) and α -glucosidase inhibitory (52.4%) effects. LC-MS showed the predominant presence of bioactive flavonoids, including epicatechin, procyanidin B5 and proanthocyanidin A2. Using a bioassay-guided approach, epicatechin was isolated from a potent fraction (DCM:MeOH:water; 7:3:0.281 v/v/v solvent system) of the ethyl acetate extract, since the fraction (fraction 5) and its sub-fraction (sub-fraction 4) had appreciable flavonoid contents, radical scavenging, anti-lipid peroxidative, α -glucosidase inhibitory and glucose uptake modulatory effects. Moreover, the isolated epicatechin inhibited α -glucosidase ($IC_{50} = 35.3 \mu\text{M}$), modulated cellular glucose uptake ($EC_{50} = 78.5 \mu\text{M}$) and inhibited LPS-induced ROS production in RAW 264.7 macrophages in a dose-dependent fashion [$IC_{50} = 18.9 \mu\text{M}$; statistically comparable ($p > 0.05$) to ascorbic acid, $IC_{50} = 9.57 \mu\text{M}$]. In a fructose/STZ model of diabetic rats, the ethyl acetate extract exerted antioxidant and glycaemic control effects by improving pancreatic histology and antioxidant status, improving insulin secretion, promoting glycogen storage and modulating cellular glucose uptake and utilization.

Conclusion

Considering this study is bioassay-guided, it is logical to conclude that chlorogenic acid strongly influences the antioxidant capacity of avocado fruit peel, while flavonoids including catechins, proanthocyanidins and procyanidins are some of the predominant constituents influencing the glycaemic control and antioxidant properties of litchi peel.

Keywords: *Avocado fruit; Diabetes; Fruit peel; Lichi fruit; Oxidative stress; Medicinal phytochemistry.*

TABLE OF CONTENTS

	PAGE
DECLARATION OF INDEPENDENT WORK	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
TABLE OF CONTENTS	vi
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
RESEARCH OUTPUT	xvii
 CHAPTER 1	 1
INTRODUCTION	1
 CHAPTER 2	 4
LITERATURE REVIEW	4
2.1 Diabetes	4
2.2 Classification of diabetes	5
2.3 Epidemiology of type 2 diabetes	6
2.4 Type 2 diabetes risk factors	7
2.4.1 Ethnicity and genetic susceptibility	7
2.4.2 Unhealthy dieting, obesity, and physical inactivity	9
2.5 Pathophysiology of type 2 diabetes	10
2.6 Relationship between oxidative stress in type 2 diabetes	11
2.7 Diabetes Complications	14
2.7.1 Microvascular complications	14
2.7.2 Macrovascular complications	17
2.8 Management of type 2 diabetes	18
2.8.1 Commercially available anti-hyperglycaemic medications	20
2.9 Fruit wastes as potential sources of bioactive phytochemicals	25
2.10 Avocado (<i>Persea americana</i> Mill.)	31
2.10.1 Antioxidant and phytochemical profile of avocado fruit	32
2.10.2 Antioxidant potential and phytochemical profile of Avocado fruit peel	35
2.11 Litchi (<i>Litchi chinensis</i> Sonn.)	35

	PAGE
2.11.1 The bioactive potential of litchi fruit in metabolic and oxidative diseases	36
2.11.2 The bioactive potential of litchi fruit peel in metabolic and oxidative diseases	37
2.12 Problem statement	43
2.13 Aim and objectives	44
 CHAPTER 3	 45
MATERIALS AND METHODS	45
3.1 Litchi fruit peel and solvent extraction	45
3.2 Screening the phenolic, flavonoid, antioxidant and glycaemic control properties of extracts	45
3.2.1 Total phenol content estimation	45
3.2.2 Total flavonoid content estimation	46
3.2.3 Ferric (Fe ³⁺) antioxidant capacity measurement	46
3.2.4 DPPH radical scavenging capacity measurement	47
3.2.5 ABTS radical scavenging capacity measurement	47
3.2.6 Nitric oxide (NO) radical scavenging capacity measurement	47
3.2.7 Measurement of linoleic acid peroxidation inhibition	48
3.2.8 Measurement of α -glucosidase inhibition	48
3.2.9 Measurement of α -amylase inhibition	49
3.3 Bioassay guided fractionation of the avocado peel methanol extract	49
3.3.1 Qualitative phytochemical profiling and antioxidant evaluation of fractions from the avocado peel methanol extract	49
3.3.2 Liquid chromatography-mass spectroscopic (LC-MS) analysis of fraction 8 from avocado peel methanol extract	50
3.3.3 Dose-dependent radical scavenging and anti-linoleic acid peroxidative assessment of fraction 8 and chlorogenic acid	50
3.3.4 Measurement of the antioxidant capacity of fraction 8 and chlorogenic acid in Chang cells	51
3.3.5 Measurement of the inhibitory effect of fraction 8 and chlorogenic acid on ROS and NO production inhibition in RAW 264.7 cells	51
3.3.6 Density functional theory (DFT) analysis	53
3.4 Bioassay-guided fractionation of litchi peel ethyl acetate extract and isolation/purification of epicatechin	53

	PAGE
3.4.1 Measurement of glucose modulatory activity in Chang cells	55
3.4.2 Nuclear magnetic resonance spectroscopy and mass spectrometry of the isolated compound	55
3.4.3 Measuring the inhibitory effect of the isolated compound on lipopolysaccharide (LPS)-induced ROS production in RAW 264.7 cells	56
3.4.4 Measuring the dose-dependent α -glucosidase inhibitory and glucose uptake modulatory effect of the isolated compound	56
3.5 IC ₅₀ and EC ₅₀ computation	56
3.6 <i>In vivo</i> antidiabetic and antioxidant assessment of litchi peel ethyl acetate extract	56
3.6.1 Animals	57
3.6.2 Diabetes induction	57
3.6.3 Treatment	57
3.6.4 Measurement of fluid intake, food intake and non-fasting blood glucose	58
3.6.5 Measurement of oral glucose tolerance	58
3.6.6 Euthanization and sampling	58
3.6.7 Measurement of liver and muscle glycogen content	59
3.6.8 Measurement of serum insulin	59
3.6.9 Tissue homogenization and protein estimation	59
3.6.10 Measurement of muscle tissue hexokinase activity	60
3.6.11 Measurement of muscle tissue Akt phosphorylation	60
3.6.12 Measurement of lipid peroxidation	61
3.6.13 Measurement of superoxide dismutase activity	61
3.6.14 Measurement of catalase activity	62
3.6.15 Histopathological examination of pancreatic tissues	62
3.6 Data and statistics presentation	62
CHAPTER 4	63
UNRAVELLING THE INFLUENCE OF CHLOROGENIC ACID ON THE ANTIOXIDANT PHYTOCHEMISTRY OF AVOCADO (<i>PERSEA AMERICANA</i> MILL.) FRUIT PEEL	63
4.1 Abstract	64
4.2 Introduction	64

	PAGE
4.3 Materials and methods	66
4.4 Results	66
4.5 Discussion	75
4.6 Conclusion	78
 CHAPTER 5	 79
EPICATECHIN ISOLATED FROM <i>LITCHI CHINENSIS</i> SONN. (LITCHI) FRUIT PEEL ETHYL ACETATE EXTRACT MODULATED GLUCOSE UPTAKE IN CHANG CELLS AND SUPPRESSED ROS PRODUCTION IN RAW 264.7 MACROPHAGES	 79
5.1 Abstract	80
5.2 Introduction	80
5.3 Materials and methods	82
5.4 Results and discussion	82
5.4.1 Phytochemical and bioactive profiles of the peel extracts	83
5.4.2 Phytochemical and bioactive profiles of the fractions and sub-fractions from the ethyl acetate peel extract	84
5.4.3 Spectrometric and bioactive profiles of the isolated compound	89
5.5 Conclusion	94
 CHAPTER 6	 95
INVESTIGATING THE PHYTOCHEMISTRY AND UNDERLYING GLYCAEMIC CONTROL MECHANISMS OF <i>LITCHI CHINENSIS</i> SONN. (LITCHI) PEEL ETHYL ACETATE EXTRACT IN A FRUCTOSE/STREPTOZOTOCIN DIABETIC MODEL OF RATS	 95
6.1 Abstract	96
6.2 Introduction	96
6.3 Materials and methods	98
6.4 Results and discussion	98
6.4.1 Phytochemical profile of litchi peel ethyl acetate extracts	99
6.4.2 Glycaemic control and antioxidant potential of litchi peel ethyl acetate	100
6.5 Conclusion	107
 CHAPTER 7	 109
GENERAL DISCUSSION AND CONCLUSION	109

	PAGE
6.1 General discussion	109
6.2 General conclusion	114
REFERENCES	116
APPENDIX	151

LIST OF TABLES

		Page
Table 2.1	Antidiabetic drugs, modes of action, and side effects.	18
Table 2.2	<i>In vitro</i> antioxidant and glycaemic control potential and phytochemical profile of wastes from fruits.	26
Table 2.3	<i>In vitro</i> antioxidant and phytochemical constituents of avocado fruit.	32
Table 2.4	Glycaemic control, antioxidant, and phytochemical profiles of the peel of litchi fruit.	40
Table 4.1	Total phenol and flavonoid contents and <i>in vitro</i> Fe ³⁺ reducing and radical scavenging activity of crude extract at 45 µg/mL	67
Table 4.2	Total phenol and flavonoid contents and <i>in vitro</i> Fe ³⁺ reducing and radical scavenging activity of the fractions from the methanol crude extract at 25 µg/mL	67
Table 4.3	LC-MS data of fraction 8 from the methanol extract of avocado peel.	68
Table 4.4	IC ₅₀ values for the dose-dependent activities of the tested samples.	70
Table 4.5	Bond dissociation enthalpy (<i>BDE</i>), adiabatic ionization potential (<i>AIP</i>), proton dissociation enthalpy (<i>PDE</i>), proton affinity (<i>PA</i>), and electron transfer enthalpy (<i>ETE</i>) obtained (in kcal/mol) of the reaction species for the capture of the DPPH radical via 5-CQA.	75
Table 5.1	Total phenol and flavonoid contents and <i>in vitro</i> Fe ³⁺ reducing, radical scavenging, anti-linoleic acid peroxidative and enzyme inhibitory activities of crude extracts at 55 µg/mL.	83
Table 5.2	Total phenol and flavonoid contents and <i>in vitro</i> Fe ³⁺ reducing, radical scavenging and enzyme inhibitory activities of fractions from the crude ethyl acetate extract at 35 µg/mL.	85
Table 5.3	LC-MS data of fraction 8 from the ethyl acetate extract of litchi peel.	86

	Page
Table 5.4 <i>In vitro</i> radical scavenging, anti-linoleic acid peroxidative, enzyme inhibitory and glucose uptake modulatory activities of sub-fractions at 20 µg/mL.	88
Table 5.5 ¹ H (400 MHz) and ¹³ C (100 MHz) NMR data of the isolated compound compared with reported literature.	90
Table 5.6 IC ₅₀ and EC ₅₀ values for the dose-dependent activities of the tested samples.	92
Table 6.1 LC-MS data of litchi peel ethyl acetate extract.	99
Table 6.2 Food intake, water intake and weekly body weight.	100
Table 6.3 Serum insulin, tissue glycogen content and muscle tissue hexokinase activity.	104
Table 6.4 Lipid peroxidation and antioxidant enzymes activity in the serum and tissue of rats.	105

LIST OF FIGURES

	Page
Figure 2.1 Types of diabetes.	5
Figure 2.2 Risk factors of type 2 diabetes.	8
Figure 2.3 Pathophysiology of type 2 diabetes.	10
Figure 2.4 The role of oxidative stress in diabetic pathologies and complications.	12
Figure 2.5 Possible molecular mechanisms underlying oxidative stress-mediated beta-cell dysfunction and insulin resistance.	13
Figure 2.6 Recovery and applications of bioactive chemicals from fruit wastes.	25
Figure 2.7 Avocado fruit and its different parts	31
Figure 2.8 Litchi fruit showing the (a) seed, (b) pericarp/peel, and (c) edible pulp.	36
Figure 2.9 Structures of bioactive compounds in the peel and seed of litchi fruit.	40
Figure 3.1 Schematic representation of the isolation of compounds from the Ethyl Acetate extract of Litchie Peel	54
Figure 4.1 LC-MS chromatogram of fraction 8 from the methanol extract of avocado peel.	69
Figure 4.2 Dose-dependent (a) DPPH radical scavenging and (b) anti-linoleic acid peroxidative activities of fraction 8 and chlorogenic acid.	70
Figure 4.3 (a) Dose-dependent anti-lipid peroxidative and (b) antioxidant enzyme modulatory activities of fraction 8 and chlorogenic acid in FS-treated Chang cells.	71
Figure 4.4 Dose-dependent inhibitory effect of fraction 8 and chlorogenic acid on ROS production in LPS-treated RAW 264.7 macrophages.	72
Figure 4.5 Dose-dependent inhibitory effect of fraction 8 and chlorogenic acid on (a) NO production in LPS-treated RAW 264.7 macrophages and (b) the viability of RAW 264.7 macrophages.	73

	Page
Figure 4.6	(a) Chemical structure of chlorogenic acid (5- <i>O</i> -caffeoylquinic acid) and its lowest-energy structural conformer; (b) the electron spin density of the optimized structures of 5- <i>O</i> -caffeoylquinic acid radicals. 74
Figure 5.1	LC-MS chromatogram of fraction 8 from the ethyl acetate extract of litchi peel. 87
Figure 5.2	Structure of (+)- <i>Epi</i> -catechin 89
Figure 5.3	Dose-dependent inhibitory effect of epicatechin (EPC) and ascorbic acid (ASC) on ROS production in LPS-treated RAW 264.7 macrophages. 91
Figure 5.4	Dose-dependent inhibitory effect of epicatechin and acarbose on α -glucosidase activity. 92
Figure 5.5	(a) Glucose concentration in the medium of cells receiving different treatments and (b) % glucose uptake of cells receiving different treatments. 93
Figure 6.1	LC-MS chromatogram of litchi peel ethyl acetate extract. 100
Figure 6.2	Weekly non fasting blood glucose (NFBG). 101
Figure 6.3	(a) Oral glucose tolerance data and (b) area under the curve. 102
Figure 6.4	Histological images of the pancreatic tissues from rats. 103
Figure 6.5	Muscle tissue (a) panAkt and phosphoAkt levels and (b) phosphoAkt/panAkt ratio of rats. 106

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfuric acid
AGEs	Advanced glycation end products
AIP	Adiabatic ionization potential
AUC	Area under the curve
BDE	Bond dissociation enthalpy
BMI	Body mass index
CAT	Catalase
CDC	Centre for disease control
DBC	Diabetic control
DFT	Density functional theory
DPPH	2,2-diphenyl-1-picrylhydrazyl
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GLUT4	Glucose transporter 4
GSH	Reduced glutathione
GSK-3	Glycogen synthase kinase 3
HAT	Hydrogen atom transfer
HbA1c	Hemoglobin A 1C
HDPE	High-dose peel extract
HFD	High-fat diet
HIF-1α	Hypoxia-inducible factor 1-alpha
HMG20A	High mobility group protein 20A
IDF	International diabetes federation
IST	Insulin signal transduction
IRS1	Insulin receptor substrate 1
JNK	Jun amino-terminal kinases
LC-MS	Liquid chromatography – mass spectroscopic
LDL	Low-density lipoprotein
LDPE	Low-dose peel extract
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDA	Methylenedioxyamphetamine
MET	Metformin
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide

NC	Normal control
NFBG	Non-fasting blood glucose
NMR	Nuclear magnetic resonance
OGTT	Oral glucose tolerance test
PBS	Phosphate buffer saline
PDE	Proton dissociation enthalpy
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PPAR	Peroxisomes proliferate-activated receptor
QE	Quercetin equivalent
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Sprague-Dawley
SET-PT	Single electron transfer followed by protein transfer
SGLT-2	Sodium glucose co-transporter-2
SOD	Superoxide dismutase
SPLET	Sequential protein loss followed by electron transfer
STZ	Streptozotocin
T2D	Type 2 diabetes
T2Ds	Thiazolidinediones
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances

RESEARCH OUTPUTS

Published articles from this thesis:

1. **Izu GO**, Mfotie Njoya E, Tabakam GT, Namboozee J, Otukile KP, Tsoeu SE, Fasiku VO, Adegoke AM, Erukainure OL, Mashele SS, Makhafola TJ, Sekhoacha MP, Chukwuma CI*. (2024). Unravelling the Influence of Chlorogenic Acid on the Antioxidant Phytochemistry of Avocado (*Persea americana* Mill.) Fruit Peel. *Antioxidants*, 13(4): 456. <https://doi.org/10.3390/antiox13040456>
2. **Izu GO**, Mapasa NP, Namboozee J, Chukwuma MS, Njoya EM, Tabakam GT, Bonnet SL, Makhafola TJ, Mashele SS, Chukwuma CI*. (2024). Epicatechin Isolated from *Litchi chinensis* Sonn. (Litchi) Fruit Peel Ethyl Acetate Extract Modulated Glucose Uptake in Chang Cells and Suppressed ROS Production in RAW 264.7 Macrophages. *Antioxidants*, 13(10): 1233. <https://doi.org/10.3390/antiox13101233>
3. **Izu GO**, Mashele SS, Chukwuma CI*. (2024). Investigating the Phytochemistry and Underlying Glycemic Control Mechanisms of *Litchi chinensis* Sonn. (Litchi) Peel Ethyl Acetate Extract in a Fructose/Streptozotocin Diabetic Model of Rats. *Nutrients*, 16(21): 3644. <https://doi.org/10.3390/nu16213644>.

Other co-authored published articles and/or submitted manuscripts during doctoral program:

1. Mashile B, Setlhodi R, **Izu GO**, Erukainure OL, Mashele SS, Makhafola TJ, Eze KC, Chukwuma CI*. (2024). Temperature-dependent extraction and chromatographic recovery and characterisation of ellagitannins with potent antioxidant and glycaemic control properties from ‘Wonderful’ pomegranate peel. *International Journal of Food Science and Technology*, 59(1): 408-424. <https://doi.org/10.1111/ijfs.16823>.
2. Setlhodi R, Mashile B, **Izu GO**, Gbashi S, Mashele SS, Bonnet SL, Makhafola TJ, Chukwuma CI*. (2024). Modeling the Influence of Extraction Temperature on the Ellagitannin and Antioxidant Profiles of “Wonderful” Pomegranate Peel Using Advanced Chemometrics Analysis. *Food and Bioprocess Technology*, 17: 83-99. <https://doi.org/10.1007/s11947-023-03104-2>
3. Setlhodi R, Mashile B, **Izu GO**, Gbashi S, Mashele SS, Bonnet SL, Makhafola TJ, Chukwuma CI*. (2023). Effect of solvent extraction on the antioxidant and phytochemical profiles of ellagitannins from "wonderful" pomegranate peel: an advanced chemometrics analysis. *European Food Research and Technology*, 249: 1807–1820. <https://doi.org/10.1007/s00217-023-04255-4>

4. Chukwuma CI*, **Izu GO**, Chukwuma MS, Samson MS, Makhafola TJ, Erukainure OL. (2021). A review on the medicinal potential, toxicology, and phytochemistry of litchi fruit peel and seed. Journal of Food Biochemistry, 45(12), e13997. <https://doi.org/10.1111/jfbc.13997>.

Conference presentation from this thesis:

1. The student attended and presented a paper titled “Studies on the antioxidant phytochemistry of *Persea americana* Mill. (Avocado) fruit peel” at the 56th Annual South African Society for Basic and Clinical Pharmacology (SASBCP) conference, held at Rhodes University from 24th to 27th of August 2023.

CHAPTER 1

INTRODUCTION

Diabetes is an evolving disease. It is a multifaceted, intricate metabolic disorder characterised by aberrant protein, fat, and carbohydrate metabolism, which leads to high blood glucose, dyslipidaemia, defects in the action and functions of key metabolic enzymes, and oxidative stress-mediated cellular and tissue damage (Oguntibeju *et al.*, 2019). The condition arises from the body's inability to produce enough insulin (a hormone that helps move blood sugar into cells where it is metabolised and turned into energy) or to react to insulin as it should (CDC, 2020). According to the 10th Edition of the IDF Diabetes Atlas, 6.7 million deaths worldwide are related to diabetes in 2021 (IDF, 2021).

The three main forms of diabetes are type 1 diabetes, type 2 diabetes (T2D), and gestational diabetes, according to the Centres for Disease Control (CDC). The main feature of type 1 diabetes is a complete lack of insulin as a result of the death of pancreatic β -cells, which can be caused by viral invasion, chemical toxicity, or the action of autoimmune antibodies (Rodrigues *et al.*, 2023). The aetiology of T2D is largely influenced by genetic and environmental variables. The disease occurs due to reduced or impaired insulin responsiveness in target tissues. Peripheral tissue insulin resistance leads to decreased circulatory glucose absorption and metabolism, which gradually deteriorates pancreatic beta cell function and causes hyperglycemia (Chukwuma *et al.*, 2018). Insulin resistance can also result to dyslipidaemia, high levels of inflammatory signalling, and increased generation of reactive oxygen species, which can reduce vascular endothelial nitric oxide, leading to increasing blood pressure (Angelidi *et al.*, 2021). The third kind of diabetes, known as gestational diabetes, affects pregnant women who have or have not previously suffered from diabetes. Usually, this kind of diabetes reduces once the baby is born. But it puts the mother and kid at risk for T2D at a later time (ADA, 2022).

Persistent elevation of blood glucose can cause oxidative damage to the vasculature, potentially leading to microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (cardiovascular) issues (Yuan *et al.*, 2020). Therefore, oxidative stress is considered a major player in the development and progression of diabetic complications. (Pourghassem-Gargari *et al.*, 2011; Yuan *et al.*, 2020). Reactive oxygen species (ROS), such as hydrogen peroxide can induce a substantial reduction in insulin signalling and glucose movement in target cells (Angelidi *et al.*, 2021). The pathophysiology of oxidant-induced insulin resistance also involves stress-activated serine kinases and excess generation of hydrogen peroxide and superoxide ions in the mitochondria, which exacerbate the development of insulin resistance in skeletal muscle (Angelidi *et al.*, 2021).

Studies have shown that diabetes is a serious health challenge throughout Africa, especially in South Africa. In addition to direct healthcare costs, diabetes leads to premature deaths and disabilities, which significantly impact the well-being of patients and caregivers (Mayosi *et al.*, 2009). The people with

low to moderate incomes are the most affected since they find it difficult to pay for high-quality medical treatment. This is because commercially available antidiabetic drugs such as the biguanides, sulfonylureas, thiazolidinedione, α -glucosidase inhibitors, dipeptidyl-peptidase IV inhibitors, insulin- and incretin-based therapies are expensive and most times unaffordable. Also, the are side effects of synthetic commercial drugs (weight gain, hypoglycaemia, gastrointestinal disturbances, urinary tract and genital infections, jaundice, hepatitis, liver injury, allergic reactions, etc.) have discouraged their use (Bösenberg & van Zyl, 2008; Chaudhury *et al.*, 2017). The rural and middle-class population relies on fruits, vegetables, and native plant mixtures as supplemental and/or alternative treatments for many chronic non-communicable disorders, such as vascular complications and diabetes (Mothibe & Sibanda, 2019).

Fruits and vegetables contain bioactive compounds that have a significant function in controlling human metabolisms, which often leads to enhanced health and overall well-being. Studies on animals and cells have documented the health benefits of phytochemicals, which frequently involve controlling cell signalling pathways, scavenging free radicals, and reducing inflammation. (Mahmoud *et al.*, 2013). Bioactive compound applications have been linked to reduced incidences or delayed progression of many diseases (Awuchi, 2021). Natural antioxidants found in fruits help reduce oxidative stress and slow the development of metabolic and cardiovascular problems associated with diabetes (Wallace *et al.*, 2019).

Some published articles have shown that non-edible wastes, particularly fruit peels, contain phenolics and other phytochemicals as in the edible portions (Sagar *et al.*, 2018, Rafiq *et al.*, 2019). These compounds could be important in addressing various chronic diseases such as diabetes, hypertension, obesity, and cardiovascular issues. This is pertinent in a country like South Africa, where substantial quantities of under-utilized fruit wastes (peels, seeds, pomace, etc) are produced annually, which potentially hold medicinal and bio-economic importance (Khan *et al.*, 2015). The waste from these fruits could be investigated as candidates for naturally occurring sources of bioactive compounds for the treatment of diseases. The peel wastes of fruits could be probed for the isolation of bioactive compounds that can prevent thrombosis, oxidative stress, osteoporosis, and diabetes, lower the risk of cardiovascular illnesses and capillary fragility and suppress platelet aggregation (Vinayagam *et al.*, 2017; Sun *et al.*, 2020). Fruits such as *Persea americana* Mill (avocado) and *Litchi chinensis* Sonn (litchi) have appreciable nutritional and phytochemical profile and may be transformed into medicines or nutraceuticals that can be used to treat and prevent many chronic illnesses, including cancer, diabetes, hypertension, hyperglycemia, hyperlipidaemia, atherosclerosis, and hypertension (Yahia & Woolf, 2011; Yao *et al.*, 2021).

The peel of avocado is a big source of phytochemicals and has higher phenolic content and antioxidant activity than the edible part (pulp) because of its three times higher polyphenol concentration (Melgar

et al., 2018; Tremocoldi *et al.*, 2018). In comparison with other tropical fruit peels (banana, melon, passion fruit, papaya, pineapple, and watermelon), the avocado dried peel has the most significant flavonoid concentration, and the fruit's raw peel has the highest overall phenolic content. (Akan, 2021). The peel extracts also act as inhibitors for the inflammation mediators and pro-inflammatory cytokines TNF- α , properties that may be attributed to a radical scavenging during inflammation (Tremocoldi *et al.*, 2018). The peel also has a potent antimicrobial property that is comparable to that of nisin, a naturally occurring antimicrobial dipeptide (Coman *et al.*, 2020).

On the other hand, several studies have shown that litchi extracts exhibit antidiabetic activity by reducing pancreatic damage (Li *et al.*, 2018). Also, litchi ameliorates insulin resistance and improves glucose tolerance by stimulating the expression of proteins involved in glucose transport (Li *et al.*, 2018). Litchi peel is a known source of Oligonol procyanidins A and low-molecular-weight oligomers such as epicatechin, catechin, and epigallocatechin gallate (Park *et al.*, 2018). These have been identified as strong phenolics that promote health through metabolism, vascularity (Fu *et al.*, 2017; Rauf *et al.*, 2019). These bioactive flavonoids have also been reported in the peel of litchi fruit (Zhao *et al.*, 2006; 2007; Chukwuma *et al.*, 2021). Feeding diabetic mice an extract containing oligonol from litchi peel decreased blood sugar levels and increased insulin release from β cells (Park *et al.*, 2018). The extract enhanced pancreatic histology and decreased oxidative stress and inflammation in the pancreas, which may have some bearing on the organ's capacity to maintain insulin output. (Park *et al.*, 2018).

Despite the antioxidant, glycaemic control, and anti-inflammatory attributes of the peel of avocado and litchi fruits, prior research has been unable to establish a connection or association between the primary phytoconstituents found in the peel of these fruits and their bioactivity. Establishing such a connection will provide further knowledge about the antioxidant and anti-hyperglycaemic phytochemistry of the peel from these fruits. Understanding the antioxidant and anti-hyperglycaemic phytochemistry of the peel of these fruits may also inform their effective therapeutic application. Using a bioassay-guided approach, the current study employed computational, *in vitro*, cell-based, and *in vivo* models to investigate the antioxidant and glycaemic control phytochemistry of the peel of avocado and litchi fruits.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes

Diabetes is a multifaceted and long-term metabolic illness with a variety of manifestations, each with a unique pathophysiological cause and mostly sharing overlapping characteristics (Banday *et al.*, 2021). The disease is marked by abnormally elevated blood glucose levels, brought on by a hereditary or acquired pancreatic insufficiency in insulin synthesis or by the incapacity of insulin to operate in target tissues and cells (ADA, 2015; Soumya *et al.*, 2011). The primary regulator of blood glucose, lipid, and protein metabolism is insulin, a peptide hormone. After food consumption, blood glucose levels rise, and the pancreatic islets of Langerhans' β -cells produce insulin to regulate blood glucose levels (Norton *et al.*, 2022).

In a diseased state, the body's cells cannot metabolize sugar efficiently, because the pancreas is unable to produce enough insulin or because the body is unable to respond to insulin efficiently. As a result, the body breaks down fat, protein, and glycogen to make glucose, leading to continuous hyperglycemia and an overabundance of liver byproducts. (Ghimire and Dhamoon, 2023). These irregularities happen gradually and eventually compromises the functioning and structure of micro- and macro vasculature throughout the body. This may lead to structural and functional abnormalities in the circulatory system of the organs, resulting in micro- and macrovascular problems (Banday *et al.*, 2021). Organs like the heart, kidneys, nerves, eyes, and kidneys are the most susceptible, resulting in their damage and/or failure (Banday *et al.*, 2021). Retinopathy is a common consequence of eye-related complications that can result in blindness. Renal failure and nephropathy can result from kidney-related complications. Coronary heart disease and hypertension are examples of heart-related complications. Peripheral and/or autonomic neuropathy can result from nerve-related complications. Short-term peripheral neuropathy can cause foot infections, including ulcers that need to be amputated, and Charcot joint (osteoarthropathy). Long-term autonomic neuropathy can cause disorders of the heart, stomach, and genitourinary system, including amputation-requiring ulcers and Charcot joint osteoarthropathy (ADA, 2018; Rawshani *et al.*, 2017).

Symptoms associated with persistent high blood glucose include excessive urination (polyuria), extreme thirst (polydipsia), weight loss, and sometimes increased appetite (polyphagia) and blurred vision. In addition, it may weaken the immune system, making individuals more susceptible to certain infections. These complications are common in both industrialized and developing nations, causing a significant socioeconomic obstacle (Subramani *et al.*, 2019; Salehi *et al.*, 2019; Chatrati *et al.*, 2022).

2.2 Classification of diabetes

Diabetes is classified by the American Diabetes Association (ADA) based on aetiology and clinical presentation (ElSayed *et al.*, 2023, ADA, 2023). **Figure 2.1** depicts the different types of diabetes, which are highlighted below (Banday *et al.*, 2021).

- 1. Type 1 diabetes:** Caused by autoimmune destruction of β -cells. This condition typically results in complete insulin insufficiency.
- 2. Type 2 diabetes:** Usually accompanied by insulin resistance and metabolic syndrome, which results in a non-autoimmune gradual loss of sufficient β -cell insulin production.
- 3. Specific types of diabetes, or secondary diabetes:** This class of diabetic disorders includes conditions caused by exocrine pancreas diseases (like pancreatitis and cystic fibrosis), drug- or chemical-induced diabetes (like glucocorticoid use, HIV/AIDS treatment, or organ transplantation), and monogenic diabetes syndromes (like neonatal diabetes and maturity-onset diabetes of the young).
- 4. Gestational diabetes:** Impaired glycaemic control in pregnant women that was not present before pregnancy but was discovered in the second or third trimester of pregnancy.

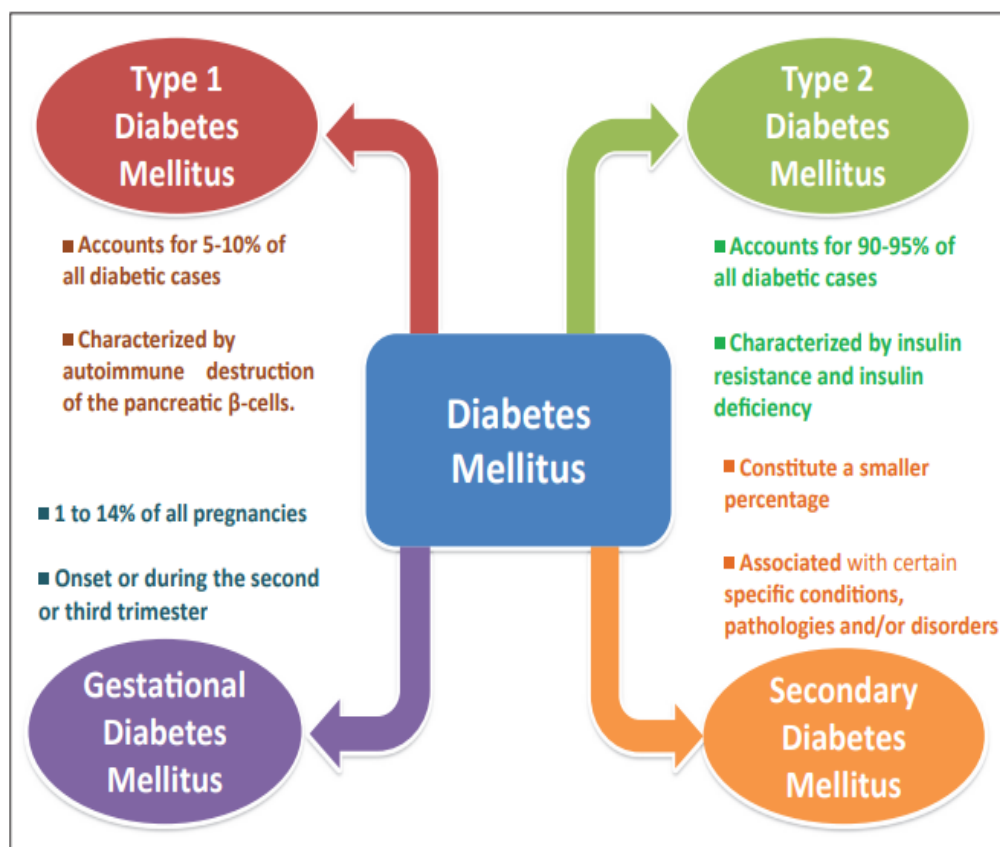


Figure 2.1: Types of diabetes (Banday *et al.*, 2021).

Type 2 diabetes (T2D) is the most common form of diabetes, which makes up more than 90% of cases (Goyal *et al.*, 2024). Hence, this review focuses on its pathophysiology and management. It is also known as non-insulin-dependent diabetes or adult-onset diabetes. T2D is characterized by insulin resistance, hyperglycaemia, and relative insulin deficiency and with time can progress to overt diabetes, due to compromised β -cell integrity and function (Galicia-Garcia *et al.*, 2020; Damanik *et al.*, 2021). The mechanism by which insulin resistance develops is complicated and can be impacted by different factors, such as growing older, being obese, having a family history of diabetes, not exercising, and embracing modern lifestyles (Zhu *et al.*, 2021). Additionally, women who have experienced gestational diabetes mellitus and individuals with underlying cardiovascular impairments may also be at increased risk (Brown *et al.*, 2009; Brown *et al.*, 2010, Banday *et al.*, 2021). The disease is slow-progressing and asymptomatic. Even with mild hyperglycaemia, it may take years to develop to an overt stage. As a result, it is often not diagnosed until classic symptoms (weight loss, growth impairment, blurred vision, polyuria, polydipsia, etc.) linked to extreme hyperglycaemia develop in the advanced stages of the disease (Banday *et al.*, 2021). For many years, there have been theories on the possibility of preventing or delaying T2D by altering certain of its risk factors. The protracted and frequently slow increase in blood sugar levels before the diagnosis of diabetes implies that precautionary measures implemented throughout that time frame may be beneficial in averting T2D (Majety *et al.*, 2023).

2.3 Epidemiology of type 2 diabetes

The global incidence of T2D is increasing with no signs of slowing down. The increasingly heavy burden on lower-income nations is a major concern (Khan *et al.*, 2019). According to a 2021 estimate from the International Diabetes Federation (IDF), 537 million adults worldwide suffered from diabetes, and is projected to rise to about 783 million adults by 2045 (IDF, 2021). Because T2D affects approximately 25% of people over 65, and this percentage is likely to increase in the coming decades, prevention efforts are critical given the detrimental human and financial impact of the disease, coupled with the difficulty of treating it once diagnosed (ADA, 2019). The elderly with T2D are more likely to experience functional disability, co-occurring illnesses, numerous common geriatric disorders, and microvascular and macrovascular complications (Kirkman *et al.*, 2012).

According to the 10th Edition of the IDF Atlas, 4.2 million persons in South Africa between the ages of 20 and 79 have type 2 diabetes (IDF, 2021). With a T2D prevalence incidence of 11.3%, South Africa has one of the highest in Sub-Saharan Africa (IDF, 2023; Sahadew & Singaram, 2019). This increase is influenced by urbanization, unhealthy lifestyle choices, obesity, poor diet, increasing fasting blood glucose, and little physical activity (Werfalli *et al.*, 2018; Pheiffer *et al.*, 2018). A study has shown that by 2030 the financial burden (i.e., direct medical expenditures) of diagnosed T2D on the public healthcare system in South Africa will amount to about 35.1 billion South African Rands (ZAR) (Erzse

et al., 2019). Moreover, the IDF reported that in 2021 about 96, 000 diabetes related fatalities in South Africa. Diabetes-related medical costs reached US\$1700 per person, reiterating the negative socio-economic impact that diabetes has in Africa and globally.

Diabetic complications, such as hypertension, hyperlipidaemia, obesity, cataracts, diabetic retinopathy, impaired renal function, and erectile dysfunction, are prominent in Sub-Saharan Africa, stressing the importance of strategies for early diagnosis and prevention (Ekoru *et al.*, 2019). Moreover, T2D plays a key role in the development and progression of cardiovascular diseases, including cardiac dysfunction and heart failure, underscoring the need for better awareness, diagnostics, and management (Glezeva *et al.*, 2018).

2.4 Type 2 diabetes risk factors

The risk factors of T2D range from non-modifiable, including ethnicity and genetic predisposition to modifiable, which are mostly lifestyle-related (Galicia-Garcia *et al.*, 2020) (**Figure 2.2**).

2.4.1 Ethnicity and genetic susceptibility

The complexity of diabetes demands a comprehensive understanding of its risk factors for effective prevention and treatment. Ethnic disparities in T2D incidence may stem from various factors, including the distribution of body fat, adipose tissue function, and insulin secretion and sensitivity. These factors are influenced by a combination of genetic and environmental factors, and thus understanding the interrelation is crucial to combat the disease (Golden *et al.*, 2019; Yaghootkar *et al.*, 2020). According to a previous report, the incidence of diabetes in the United States displays a significant variation based on self-reported ethnicity and physician-diagnosed diabetes. This includes 17.7% of white European individuals, 22.5% of Asians, 30.6% of Hispanics and Africans, and 45.2% of American Indians/Alaska, who are 75 years of age or older (Golden *et al.*, 2019). The diversity is also noticeable within ethnic sub-groups, where Indians have the highest risk of disease (38.8%) among Asians, and Mexicans have the highest prevalence (33.4%) among Hispanics (Cowe *et al.*, 2018).

In South Africa, the coloured population exhibits a higher occurrence of T2D in comparison to the Indian and Black populations (Pheiffer *et al.*, 2021). Although the precise causes are yet to be fully understood, experts have put forward potential factors, such as genetic predisposition and gene-environment interplay, socioeconomic advancement, and contemporary behaviours that encourage weight gain (Hu, 2011; Basu *et al.*, 2013). Studies have demonstrated that an individual's genetic makeup greatly influences their susceptibility to T2D. As a result, pinpointing the genetic variations that contribute to the illness is critical in effectively managing the condition. This approach could

enhance the patient's overall health status, and minimize the likelihood of complications (Sirdah *et al.*, 2020). Genome-wide association studies (GWAS) have pointed out over 500 genomic variants and susceptibility genes (*TCF7L2*, *PPARG*, *CDKAL1*, *CDKN2A/B*, *IGFBP2*, *KCNJ*, *CAPN10*, *FTO*, and others) associated with increased risk to T2D (Vujkovic *et al.*, 2020; Xue *et al.*, 2018). Most of these variants affect insulin secretion, by either directly altering islet function (e.g. *KCNJ11*) and its development (*HNF1A*, *WFS1*) or indirectly by altering incretin signalling (*GLP1R*) (Dimas *et al.*, 2014).

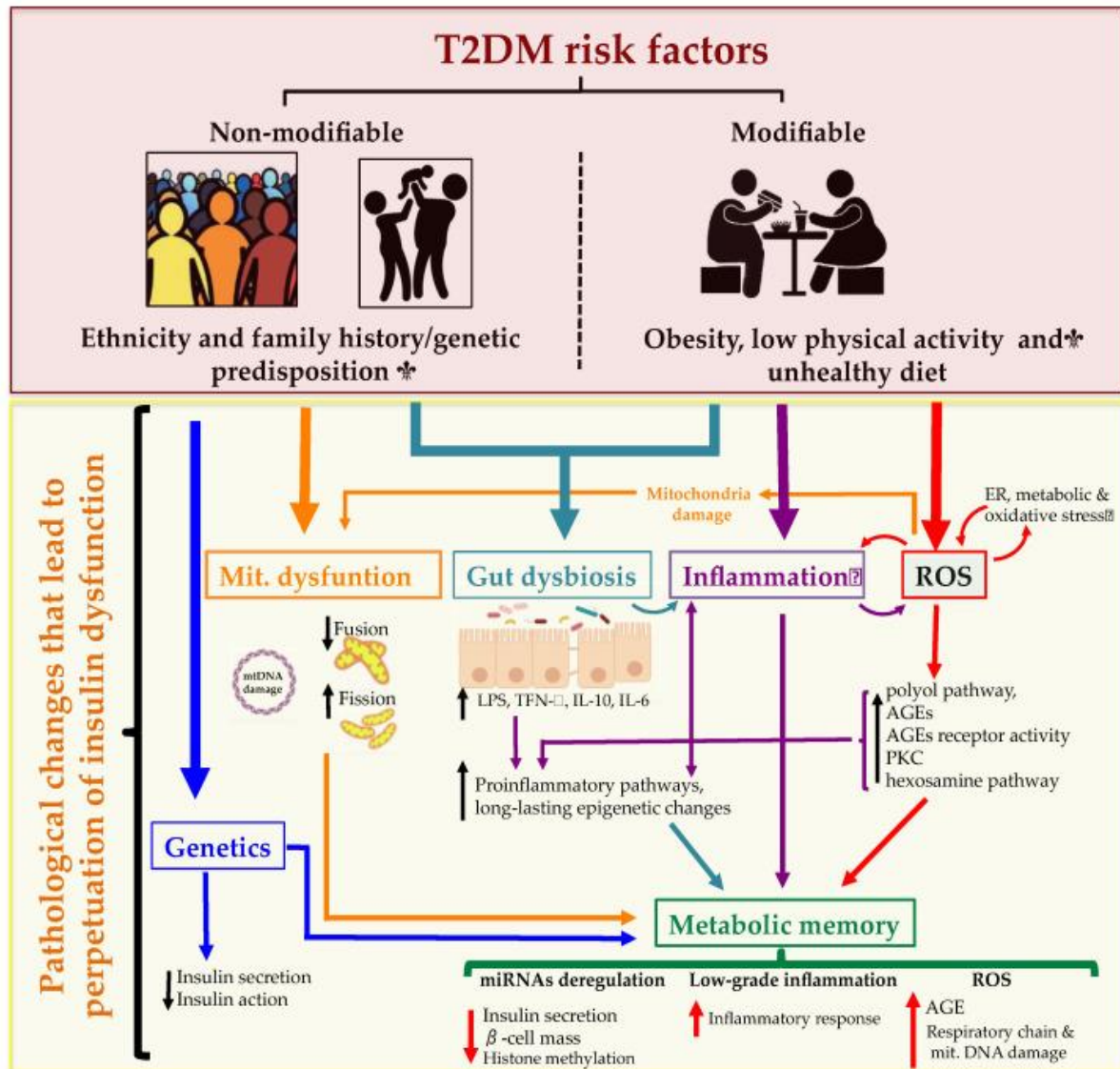


Figure 2.2: Risk factors of type 2 diabetes (Galicia-Garcia *et al.*, 2020).

Conversely, a small number of loci linked to T2D appear to function predominantly through insulin resistance (*PPARG*, *IRS1*), adipogenesis (*KLF14*, *PPARG*), and obesity (*FTO*) (Dimas *et al.*, 2014). Additionally, research suggests that genetic diversity can be used to predict the occurrence of T2D (Vassy *et al.*, 2012; Imamura *et al.*, 2013; Talmud *et al.*, 2015; Mahajan *et al.*, 2018). Genetic data can

aid in projecting lifetime risk. However, maintaining a normal body weight can mitigate a high genetic risk (Ligthart *et al.*, 2021). Specific genetic variations, including IGF2BP2, HMG20A, and HNF1B, can heighten the risk of T2D, especially among the Han Chinese population (Huang *et al.*, 2020). In 2020, a comprehensive study delved into the genetic predisposition to T2D through a meta-analysis of over 228,000 cases and 1.1 million controls across several studies, including the Million Veteran Program (MVP), DIAMANTE, and Biobank Japan. The study revealed 318 newly discovered risk loci associated with T2D, including some that are connected to associated vascular outcomes or complications (Vujkovic *et al.*, 2020). In another study by Goyal *et al.* (2019), the influence of genetic variants of HHEX, CDKAL1, WFS1, and SLC30A8 genes on different Asian populations was discussed, highlighting the heightened vulnerability of Asians to these variants (Goyal *et al.*, 2019). The complex interaction between hereditary and environmental variables in the likelihood of acquiring T2D was emphasized by the above studies taken together. Therefore, to properly manage T2D, it is important to determine the particular genetic variations that contribute to the condition. This strategy may improve clinical results for patients.

2.4.2 Unhealthy dieting, obesity, and physical inactivity

The development of T2D can be influenced by an unhealthy diet, obesity, and physical inactivity (Zhu *et al.*, 2021; Oynotkinova *et al.*, 2022). Obesity, especially in the abdominal area [shown by a body-mass index (BMI) ≥ 30 kg/m²], can significantly increase one's chances of developing T2D, this is due to metabolic abnormalities that lead to insulin resistance and disruptions in glucose metabolism (Feng *et al.*, 2021; Ruze *et al.*, 2023). Increased synthesis of pro-inflammatory adipokines from fat tissue (including interleukin-6 and tumour necrosis factor-alpha) in the vital bodily processes that modulate insulin resistance is a major culprit, which is caused by obesity. Insulin resistance may result as these adipokines disrupts the insulin signalling pathways (Eckel *et al.*, 2011). The body then produces more insulin in response to insulin resistance, which results in the build-up of extra fat in the liver. Fat that builds up in the liver is sent to other organs, such as the pancreas, and over time, this can cause the secretion of insulin to gradually decrease. This vicious cycle of increased insulin production and ectopic fat storage leads to hyperglycaemia and the onset of T2D (Sulu *et al.*, 2024). Adopting appropriate physical activity patterns either alone or in combination with diet and drug therapy (such as oral hypoglycaemic agents) can significantly reduce the risk of T2D. Additionally, losing weight can cause the pancreas, liver, and other peripheral organs to rapidly lose ectopic fat, which can significantly improve insulin resistance. Moreover, a lower body weight might increase pancreatic first-phase insulin production, which is crucial for preventing the development of T2D. This can improve glycaemic control and even aid in treating T2D (Taylor *et al.*, 2018; Al-Mrabeh *et al.*, 2020; Zhyzhneuskaya *et al.*, 2020). Therefore, incorporating healthy lifestyle changes, such as a nutritious diet and regular exercise

is essential to mitigate risk factors for T2D and cardiovascular diseases (Sigal *et al.*, 2004; Gao *et al.*, 2022; Oynotkinova *et al.*, 2022).

2.5 Pathophysiology of type 2 diabetes

Type 2 diabetes is a metabolic disorder marked by defects in insulin secretion, insulin resistance, and eventual failure of pancreatic β -cells. High blood sugar levels are the outcome of this condition, which is preceded by a reduction in insulin-mediated glucose transport into the liver, muscle, and fat cells. Elevated blood sugar levels have the potential to alter both major and tiny blood capillaries across the body. Over time, the excess glucose in the blood can lead to an increased rate of fat breakdown, which can contribute to the progression of the disease and the onset of serious complications such as cardiovascular diseases, cerebrovascular diseases, retinopathy, neuropathy, and renal diseases (Nowotny *et al.*, 2015; Zheng *et al.*, 2018). **Figure 2.3** shows the pathophysiological pathways of T2D.

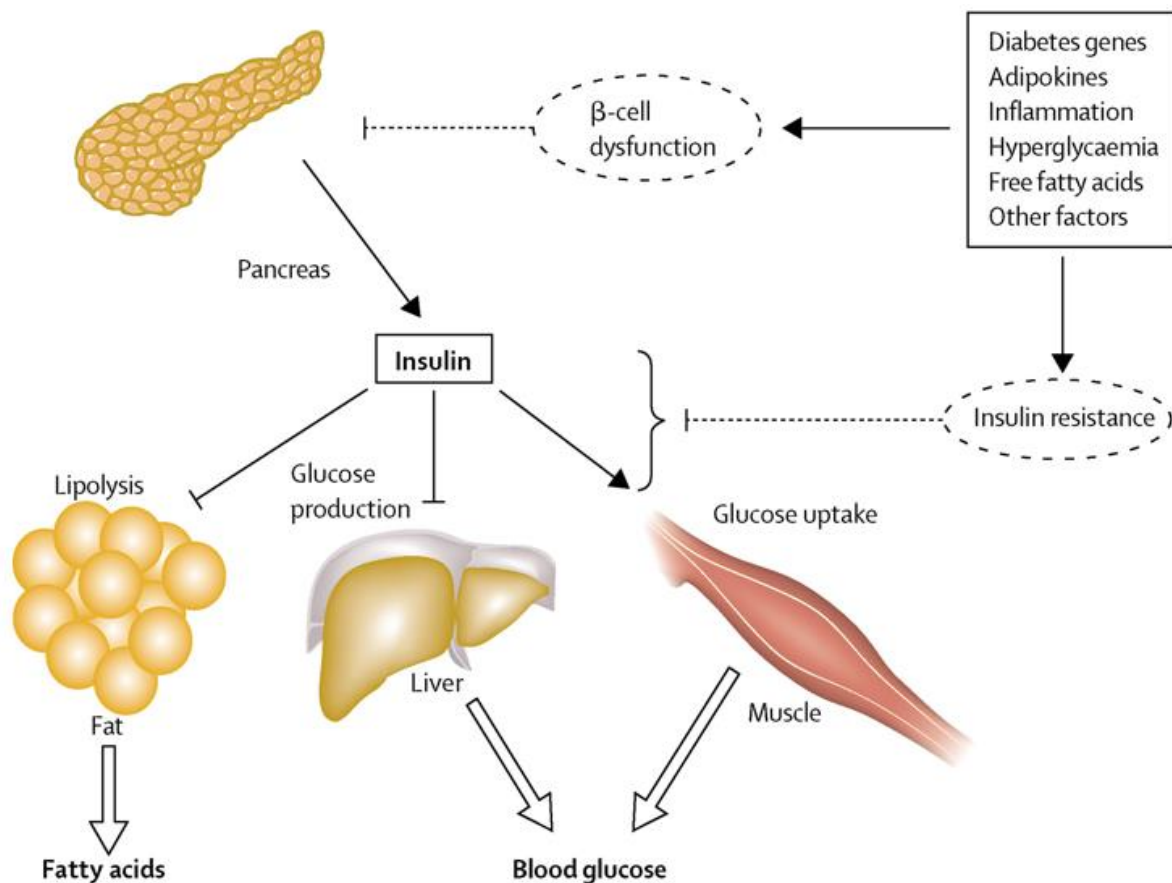


Figure 2.3: Pathophysiology of type 2 diabetes (Stumvoll *et al.*, 2005).

Impaired insulin production and insulin resistance are both critical components in the development of T2D. Patients suffering from these disorders are usually obese and usually develop them in adulthood; the frequency rises with aging and the loss of β -cell activity. Insulin release is decreased when β -cells

are not working properly, which restricts the body's capacity to maintain appropriate glucose levels (Stumvoll *et al.*, 2005). Insulin resistance also lowers the quantity of glucose taken by muscle, the liver, and adipose tissue while increasing the liver's production of glucose. While T2D is a result of both processes, β -cell malfunction often manifests as a more severe condition than insulin resistance (Cerf, 2013; Zheng *et al.*, 2018). However, when both β -cell dysfunction and insulin resistance are present, hyperglycaemia worsens, leading to overt T2D. Other than pro-inflammatory response and oxidative stress, β -cell dysfunction could be caused by exhaustion resulting from hyperinsulinemia - an increased insulin production to compensate for insulin resistance (Galicia-Garcia *et al.*, 2020).

Obesity and insulin resistance are connected to the emergence of T2D in several ways. The increased production of proinflammatory adipokines from adipose tissue, such as interleukin-6 and tumour necrosis factor-alpha can cause disruptions in the insulin signalling pathways (Eckel *et al.*, 2011). Compensatory hyperinsulinemia exacerbates hepatic fat formation, which can result in the buildup of "ectopic fat" in the pancreas and other tissues (Larson-Meyer *et al.*, 2011). This may eventually lead to a progressive reduction in insulin production and the onset of T2D. Additionally, mitochondrial dysfunction can impair insulin sensitivity and β -cell function, another mechanism that connects obesity to T2D (Bournat *et al.*, 2010). However, the critical role of oxidative stress in the pathophysiology and complications of T2D cannot be overemphasized.

2.6 Relationship between oxidative stress in type 2 diabetes

The overproduction of different reactive oxygen species (ROS) caused by the excessive rise in glucose levels in diabetes is what causes tissue damage via multiple signalling pathways. (Bhatti *et al.*, 2022). This condition can lead to various complications, including lipid peroxidation, DNA damage, and mitochondrial dysfunction, which can impede insulin signal transduction (IST) and increase the risk of insulin resistance (Yaribeygi *et al.*, 2020). Oxidative stress arises from a disparity between the production of pro-oxidant species and the body's capacity to neutralize them through antioxidant mechanisms. Oxidative stress is a notable underlying factor in the development and advancement of T2D (Azarova *et al.*, 2023). Oxidative stress can mediate the oxidative damage β -cells, thus compromising their number and function. It can also cause cell apoptosis, leading to the death and loss of β -cells in the pancreas. Pancreatic β -cells are more susceptible to oxidative damage because of low amounts of antioxidant enzymes such as catalase, superoxide dismutase, glutathione-S-transferases, glutathione peroxidase, glutathione reductase, and high endogenous generation of ROS. (Eguchi *et al.*, 2021). **Figure 2.4** shows the function of oxidative stress in T2D-related pathologies and complications.

consequently advancing the progression of T2D (Archuleta *et al.*, 2009; Stuart *et al.*, 2014) (**Figure 2.5**).

Additionally, these pathways can activate toll-like receptors (TLRs), which can hinder the function of beta cells. This worsens insulin resistance and further helps to the development of T2D (Liu *et al.*, 2023). Oxidative stress induced by ROS affects the movement of insulin-induced glucose transporter 4 (GLUT4) from the cytoplasm to the plasma membrane in adipocytes (Hurrle *et al.*, 2017). Furthermore, high levels of ROS can lead to the degradation of GLUT4, thus decreasing glucose uptake (Hurrle *et al.*, 2017). GLUT4 controls the uptake of glucose by insulin-independent cells, including, myocytes, and adipocytes. Decreased levels of GLUT4 exacerbate insulin resistance in peripheral tissues and hyperinsulinemia, which contributes to hyperglycaemia (Ma *et al.*, 2014). Therefore, regular expression and localization of GLUT4 in these tissues are important to maintain insulin sensitivity (Hurrle *et al.*, 2017).

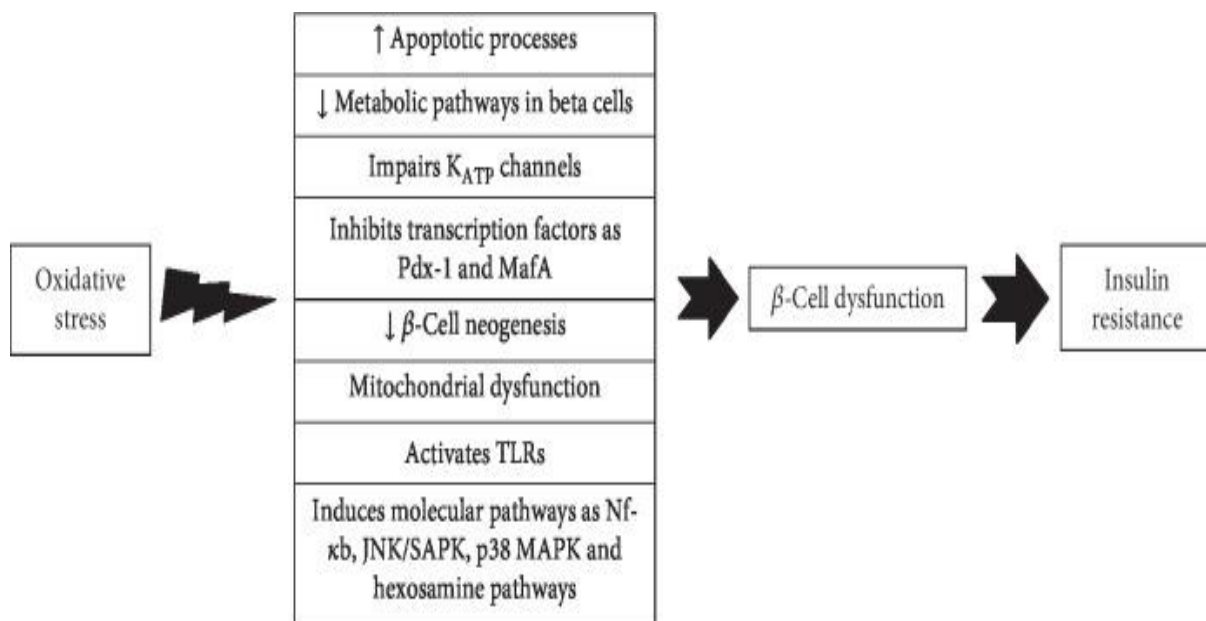


Figure 2.5: Possible molecular mechanisms underlying oxidative stress-mediated beta-cell dysfunction and insulin resistance (Yaribeygi *et al.*, 2020).

Research has also demonstrated that increased ROS stimulates casein-kinase 2 (CK-2), which activates retromers to start the trans-Golgi network and facilitates GLUT 4 translocation to lysosomes rather than the plasma membrane (Rudich *et al.*, 1999). By inhibiting transcription factors like PPAR- γ (peroxisome proliferator-activated receptor γ), CEB/Ps (CCAAT-enhancer-binding proteins), NF-1 (nuclear factor-1), p85, HIF-1 α (hypoxia-inducible factor α), MEF2 (myocyte enhancer factor 2), Nf- κ B, and microRNAs (miR-21a-5p, miR-222-3p, miR-133b-3p, miR-10b, miR-106b-5p, miR-29c-3p, and miR-133a-3p) that contribute to GLUT4 expression, oxidative stress inhibits GLUT4 transport and expression (She & Mao, 2011). Therefore, the reduction of GLUT4 expression/localization is one of

the main molecular mechanisms by which oxidative stress induces insulin resistance and promotes the occurrence of T2D (Hurrell & Hsu, 2017).

Furthermore, free radicals can cause serine phosphorylation of IRS 1 and 2, resulting in IST disease. Through the activation of the JNK/SAPK signalling pathway, free radicals can promote serine phosphorylation (Ser 307) of IRS-1, hence limiting normal IST. Additionally, through a biochemical mechanism that depends on the suppression of p38 MAPK, free radicals can suppress normal IST. Normal IST was restored in both *in vitro* and *in vivo* experimental models by suppressing the activation of the aforementioned pathways of free radicals (Aguirre *et al.*, 2000; Maddux *et al.*, 2001). Oxidative stress caused by elevated glucose levels can trigger several stress-sensitive serine/threonine kinases, such as IKK- β , and thus phosphorylating several targets, including IRS1, IRS2, and IRs. This might lead to negative consequences later on, such as decreased PI3K activation and insulin resistance. It has been shown that salicylates, an inhibitor of IKK- β , restored normal IST in oxidative stress *in vitro* (Kim *et al.*, 2001).

Serine/threonine kinases, such as AKT, GSK-3, AMPK, and mTOR, are highly sensitive to oxidative stress and may damage IST, leading to impaired insulin sensitivity, insulin resistance, and T2D (Evans *et al.*, 2003; Rains *et al.*, 2011). High levels of oxidants can cause oxidative DNA damage in T2D patients who undergo peritoneal dialysis (Pazarín-Villaseñor *et al.*, 2023). Moreover, the accumulation of AGEs and oxidative stress in T2D patients contributes to bone fragility, which increases their risk of fractures (Cavati *et al.*, 2023). The production of ROS in diabetes causes cellular impairment and damage to macromolecules such as proteins, lipids, and DNA, contributing to the pathogenesis of diabetic complications (Gonzalez *et al.*, 2011).

2.7 Diabetes Complications

Type 2 diabetes is linked to both macrovascular and microvascular complications (Viigimaa *et al.*, 2020). These complications include coronary artery disease, cerebrovascular disease, peripheral artery disease, diabetic microangiopathy, diabetic nephropathy, diabetic retinopathy, and diabetic neuropathy (Satyam *et al.*, 2021; Paul *et al.*, 2020). The prevalence of these complications is high, particularly in patients with elevated HbA1c levels (Satyam *et al.*, 2021). The complications' molecular mechanisms involve vascular endothelial dysfunction, exacerbated by chronic hyperglycaemia (Paul *et al.*, 2020).

2.7.1 Microvascular complications

Chronic intracellular hyperglycaemia and genetic predisposition eventually affect the microvasculature, leading to complications, including retinopathy, neuropathy, and nephropathy (Faselis *et al.*, 2020).

Among individuals affected by diabetes, retinopathy accounts for 13% of microvascular complications, with neuropathy and nephropathy following at 4% and 3%, respectively (Ogurtsova *et al.*, 2022).

Diabetic nephropathy

Diabetic nephropathy is a complex condition of T2D with multiple contributing factors. Hyperglycaemia, genetic predisposition, hypertension, dyslipidaemia, and proteinuria all have a critical role in its pathogenesis (Long *et al.*, 2011; Vogt *et al.*, 2018). It develops in around 40% of patients who have been diagnosed with T2D and is the primary cause of end-stage renal disease, which is linked to morbidity and mortality incidences (Beckman *et al.*, 2016, Alicic *et al.*, 2017; Maggiore *et al.*, 2017). The pathogenesis of diabetic nephropathy is brought on by an increase in the generation of free radicals, accumulation of AGEs, activation of PKC, and distortion of renin-angiotensin-aldosterone system (Koch *et al.*, 2020). Hyperglycaemia causes the high formation of AGEs and a proinflammatory state, causing a gradual decline in the glomerular filtration rate and leading to vascular complications (Faselis *et al.*, 2020; Selby *et al.*, 2020). The appearance of albuminuria is linked to a gradual reduction in the glomerular filtration rate, which corresponds with the extent of albumin excretion or increased urine protein levels, blood pressure, and blood glucose levels (Rayego-Mateos *et al.*, 2020). However, a reduction in the glomerular filtration rate may also be the result of other factors such as being female, obese, or having hypertriglyceridemia.

Inflammation plays an important role in the growth and advancement of diabetic nephropathy, with specific inflammatory mediators and intracellular signalling pathways being implicated (Skena *et al.*, 2005). The modulation of the inflammatory response has recently become known as a promising approach to mitigate diabetic kidney disease (Wada *et al.*, 2016; Niewczas *et al.*, 2019). These therapeutic methods involve inhibiting adhesion molecules, chemokines, cytokines, immune cells, and intracellular signalling pathways involved in inflammatory responses. (Rayego-Mateos *et al.*, 2020).

Diabetic retinopathy

Diabetic retinopathy is the most common complication of diabetes and the main cause of blindness in diabetic patients (Whitehead *et al.*, 2018). It is a neurovascular disease characterized by neuronal and vascular abnormalities (Oshitari, 2021). These abnormalities, including retinal vascular changes, are driven by hyperglycemia and the subsequent overproduction of ROS (Wu, 2022). The disease presents blurred vision, decreased vision, and blindness (Antonetti *et al.*, 2012). Diabetic retinopathy is divided into two types, namely non-proliferative diabetic retinopathy and proliferative diabetic retinopathy (Nikhil *et al.*, 2019). The non-proliferative diabetic retinopathy, also called early diabetic retinopathy, can be identified by clinical symptoms such as retinal microaneurysms, cotton wool spots,

haemorrhages, and exudates, which may go unnoticed by the patient. Over time, non-proliferative diabetic retinopathy can advance to a more severe form of the disease. On the other hand, proliferative diabetic retinopathy or late diabetic retinopathy is usually distinguished by the development of new blood vessels that are delicate and can rupture and bleed heavily (Solomon *et al.*, 2017).

According to Kusahara *et al.* (2018), the pathogenesis of diabetic retinopathy is complicated and includes retinal neovascularisation and the disruption of the blood-retina barrier. Several stress factors lead to diabetic retinopathy. ROS are produced by cellular metabolism, activation of the hexosamine and polyol pathways, overexpression of PKC, and AGE deposition (Biswas *et al.*, 2023) can cause retinal malfunction, cell apoptosis, lipid peroxidation, mitochondrial impairment, which can disrupt the blood barrier, and impair neural tissue homeostasis (Volpe *et al.*, 2018; Rinaldi *et al.*, 2021). The onset and development of diabetic retinopathy might include elevated oxidative stress, inflammation, and renin-angiotensin system dysfunction (Zhan, 2023).

In addition to oxidative stress, retina inflammation may occur (Wei *et al.*, 2022). Chronic inflammation plays an important part in developing diabetic retinopathy. Inflammation is caused by factors such as leukocyte arrest, inflammation-related cytokines (such as interleukin-1 β), miRNA regulation, and the activation or inhibition of signalling pathways. Microvascular lesions are common in diabetic retinopathy, and chronic inflammation contributes to the molecular pathogenesis of the disease (Spencer *et al.*, 2020; Wei *et al.*, 2022). Chronic hyperglycaemia and hypoxia stress can cause vascular endothelial growth factor (VEGF) to become highly activated in the tissues, leading to vasopermeability and proliferative diabetic retinopathy. In addition, hyperglycaemia can induce VEGF expression in microglia through the ERK1/2-NF- κ B signalling pathway. As a result, reactive microglia are stimulated by chronic hyperglycaemia and are associated with the progression of diabetic retinopathy (Semeraro *et al.*, 2019; Zhang *et al.*, 2019). Other cytokines, such as TNF- α , interleukin-6 (IL-6), and growth factors also play a role in this process. One of the most important pro-inflammatory cytokines found in diabetic retinopathy patients' vitreous is IL-6. Retinal endothelial cells' oxidative stress can be considerably decreased and retinal oxidative damage can be avoided by inhibiting IL-6 trans-signalling (Robinson *et al.*, 2020).

Diabetic neuropathy

Diabetic neuropathy is a neurodegenerative disorder that encompasses numerous factors that impact peripheral nerves. It is characterized by abnormal glucose metabolism, leading to a range of symptoms that can lead to a decrease in sensation (Kaur *et al.*, 2023). These symptoms include numbness, burning, electric-shock-type pain, aching, scratching, and cold pain (Gylfadottir *et al.*, 2019). Nerve damage may develop in individuals with long-term diabetic conditions, followed by hyperalgesia and allodynia (Beadon *et al.*, 2020). There are two main types of diabetic neuropathy, namely painless and painful.

Painless diabetic neuropathy is characterized by a lack of pain sensitivity, which can cause foot ulcers. On the other hand, painful diabetic neuropathy is characterized by neuropathic pain that can be highly distressing (Gylfadottir *et al.*, 2019). diabetic neuropathy affects both the autonomic and somatic peripheral nerves. Since autonomic innervation is present throughout the body, it affects various systems in the body such as the cardiovascular, urogenital, gastrointestinal, pupillomotor, thermoregulatory, and sudomotor systems (Javed *et al.*, 2015).

Several metabolic pathways have been implicated in diabetic neuropathy, including the polyol, hexosamine biosynthetic, and PKC pathways and the overproduction of AGE. More often than not, ROS damage and mitochondrial dysfunction may serve as their terminal pathway (Yaowei *et al.*, 2024). Persistent high blood glucose is the main influencer of the oxidative damage underlying diabetic neuropathy (Pang *et al.*, 2020). This is mediated through polyol, hexosamine, AGE, and PKC pathways, which could lead to direct nerve damage. The polyol pathway also depletes the quantity of NADPH, resulting in oxidative damage within nerve cells (Thakur *et al.*, 2021). Hyperglycaemia-induced mitochondrial flow of glucose alters energy metabolism resulting in elevated superoxide ion by-products and leakage of ROS (Eftekharpour and Fernyhough, 2022).

2.7.2 Macrovascular complications

Macrovascular complications in diabetes are caused by the narrowing of large arteries and veins due to atherosclerosis. As a result, the function of large blood vessels is primarily affected, leading to the development of atherosclerosis and other cardiovascular diseases and adversely impact the individual's health and well-being (Brownrigg *et al.*, 2013; Ferranti *et al.*, 2014).

Coronary artery disease

Coronary artery disease is a macrovascular complication that is frequently linked to diabetes. This interaction has several aspects and integrates multiple key processes. Diabetes accelerates the development of atherosclerosis, which starts when lipoproteins are deposited in the artery wall. Foam cells proliferate and low-density lipoprotein (LDL) particles oxidize in the subendothelial region, which ultimately results in vascular alterations. Acute coronary and cerebrovascular syndromes arise from the unstable rupturing of arterial plaque deposits (Bergheanu *et al.*, 2017; Bancks *et al.*, 2020). These plaques may obstruct the supply of blood to the heart, causing angina pectoris or, in more serious cases, myocardial infarction. People with diabetes have an increased risk of myocardial infarction. Numerous variables, including inflammation, endothelial dysfunction, and atherosclerosis, are responsible for this elevated risk (Zakir *et al.*, 2023; Ferguson *et al.*, 2013).

Peripheral artery disease

Peripheral artery disease is a known macrovascular diabetic complication. The process starts with atherogenesis and progresses to obstruction and reduction of blood circulation to the lower extremities (Zakir *et al.*, 2023). Numerous pathogenetic pathways, such as inflammation, aggregation of platelets, endothelial dysfunction, and vascular smooth muscle cell dysfunction have been linked to the atherosclerosis development (ADA, 2003).

Since persistent hyperglycaemia is central to developing complications of diabetes, preventing or reducing persistent high blood glucose is key in preventing the onset of most diabetic complications. While many anti-diabetic drugs act to reduce blood glucose, their long-term effects are mitigating the development of hyperglycaemia-induced complications.

2.8 Management of type 2 diabetes

Early diagnosis and intervention are crucial for reducing the likelihood of long-term secondary problems of diabetes (Xianjun *et al.*, 2018). When treating T2D, it is crucial to focus not only on reducing blood sugar levels but also on preventing the development of complications. In addition to maintaining a healthy, low-calorie diet and engaging in regular physical activity, treatment plans should be tailored to assist overweight diabetics in achieving their ideal weight. Microvascular complications decrease with lowering HbA1c. However, for prompt glycaemic control, individuals with T2D use hypoglycaemic drugs to reduce blood glucose levels. The known classes of drugs for T2D shown in **Table 2.1** below. These drugs can be taken as monotherapies or combine therapies.

Table 2.1: Antidiabetic drugs, modes of action, and side effects.

Class of drug	Examples	Action	Side effects
Biguanides	Metformin	HbA1c ↓	Gastrointestinal
		Body weight ↓→	disorders ↑ Reversible
		Cancer ↓	vitamin B12
		Cardiovascular ↓	deficiency ↑ Lactic
Glinides	Repaglinide	HbA1c ↓	Acidosis ↑
	Nateglinide	Body weight ↑	Hypoglycemia ↑
			Headache ↑ Upper
Alpha-Glucosidase inhibitors	Acarbose	HbA1c ↓	respiratory tract
		Body weight ↓ →	infection ↑
			Gastrointestinal
			disorders ↑

			Serum transaminases (AST, ALT) ↑
SGLT2-I	Empagliflozin	HbA1c ↓	Diabetic ketoacidosis
	Dapagliflozin	Body weight ↓	↑
	Canagliflozin	BP ↓	Genital infection ↑
		MACE ↓	Urinary tract infection
		Hospitalization for HF	↑
		↓	Hypovolemia ↑
		Progression of renal	Acute kidney injury ↑
		disease ↓	(related to hypovolemia)
			Canagliflozin:
			Amputation ↑
			Bone fracture ↑
Thiazolidinediones	Pioglitazone	HbA1c ↓	Body weight ↑
		BP ↓	Peripheral edema ↑
		NAFLD ↓	Anemia ↑
		MACE ↓	Hospitalization for HF
			↑
			Bone fracture in
			women ↑
DPP-4	Sitagliptin	HbA1c ↓	Saxagliptin:
	Saxagliptin		Hospitalization for HF
	Alogliptin		
Sulfonylureas	Glimepiride	HbA1c ↓	Body weight ↑
	Gliclazide		Hypoglycemia ↑ Lack
	Glibenclamide		of durable effect
	Glipizide		
GLP-1 RA	Liraglutide	HbA1c ↓↓	Gastrointestinal
	Dulaglutide	Body weight ↓	disorders ↑
	Semaglutide	Systolic BP ↓	Semaglutide:
	Orforglipron	MACE ↓	Macular edema

Combination therapy	Tirzepatide	Quality of life for
	Retatrutide	patients with HF ↑
		NAFLD ↓ →

Adopted without permission from Weinberg Sibony *et al.* (2023). Annotations: ‘↑’, Aggravation/increase; ‘↓’ improvement/decrease; “→”, not confirmed. Abbreviations: “ALT”, alanine transaminase; “AST”, aspartate aminotransferase; “BP”, blood pressure; “HbA1c”, glycated haemoglobin; “HF”, heart failure; “MACE”, major adverse cardiovascular events; “NAFLD”, non-alcoholic fatty liver disease.

2.8.1 Commercially available anti-hyperglycaemic medications

Pharmaceutical therapy should start when lifestyle changes and frequent exercise are not enough to establish appropriate metabolic control (Masood, 2022). The primary objective of these medications is to improve glycaemic control and mitigate the risk of cardiovascular damage (Chaudhury *et al.*, 2017).

Insulin sensitizers

Biguanides (Metformin)

Biguanides are the most commonly prescribed first-line treatment and the primary oral drug of choice for managing T2D in all age groups (Giaccari *et al.*, 2021). Biguanides suppress glucose production in the liver, increase sensitivity and responsiveness of cells to glucose, and decrease the digestion of glucose from the intestine (Giaccari *et al.*, 2021). Metformin is a known biguanide that can be taken with or without food, and its daily dose ranges from 500 to 2550 mg (Züleyha *et al.*, 2023). Its advantages include inexpensive cost, gentle side effects, low risk of hypoglycaemia, well-tolerated nature, and small reduction in body weight (Lv *et al.*, 2020). Metformin also suppresses hepatic gluconeogenesis and lipogenesis by increasing the activation of the AMP protein kinase enzyme. Its effects on the gastrointestinal system and GLP1 action cause a decrease in appetite and, consequently, moderate weight loss (up to 6 kg) (Giaccari *et al.*, 2021). It has cardiovascular protective properties, including regulation of blood lipids, as well as reduction of oxidative stress, blood pressure, and platelet aggregation (Erdin *et al.*, 2023). A recently published meta-analysis suggested that metformin may improve kidney function and mitigate hyperglycaemia-mediated kidney damage. Metformin may decrease the incidence of neurodegenerative conditions, and perhaps, have anti-neoplastic properties (Rutledge *et al.*, 2022).

Thiazolidinediones (Pioglitazone)

Thiazolidinediones (TZDs), also called Glitazones act by ameliorating insulin resistance and improving insulin sensitivity. They improve peripheral insulin sensitivity and reduce the liver's synthesis of glucose

(Yamanouchi, 2010). TZDs activate peroxisome proliferator-activated receptor gamma (PPAR- γ) in the cell nucleus. Activation of PPAR- γ increases insulin sensitivity in adipose tissue, skeletal muscle, and the liver. The most used form of TZDs is pioglitazone. Some therapeutic outcomes of TZDs administration include improved glycaemic control, preserved pancreatic β -cell function, positive impacts on lipid profiles and cardiovascular health, and reduced inflammation (Long *et al.*, 2022). TZDs are metabolized in the lung and excreted with bile. They do not cause hypoglycaemia, are often widely accepted, reduce secondary cardiovascular events, and lower blood pressure (DeFronzo *et al.*, 2019). They reduce insulin resistance by acting primarily on adipose tissue. Glitazones have been shown to significantly reduce the risk of T2D, even more so than metformin. However, the use of glitazones in prediabetic patients is discouraged because of possible adverse effects, such as fluid retention, weight gain, heart failure, hepatotoxicity, osteoporotic fractures, and bladder cancer (Kernan *et al.*, 2016).

Insulin secretagogues

Sulfonylureas

Sulfonylureas are a type of medication that helps to reduce blood sugar levels by causing β -cells to secrete more insulin. They achieve this by obstructing the potassium ATP channels (DeFronzo *et al.*, 2019). Additionally, they reduce gluconeogenesis in the liver. They also decrease lipolysis and the clearance of hepatic insulin (Chaudhury *et al.*, 2017). Sulfonylureas are known to have high efficacy in lowering blood glucose levels (Davies *et al.*, 2022). However, they don't have a long-lasting impact and are linked to hypoglycaemia and weight gain (Davies *et al.*, 2022). There is evidence of reduced risk of microvascular problems associated with the use of sulfonylureas (Chaudhury *et al.*, 2017). However, sulfonylureas may cause hypoglycaemia because they stimulate insulin secretion in a glucose-independent manner (Chaudhury *et al.*, 2017). Sulfonylureas are usually recommended as second-line treatment in the management of T2D (ADA, 2016). Sulfonylurea monotherapy does not correlate with a higher risk of myocardial infarction in T2D patients compared to metformin alone. Second-generation sulfonylureas, such as glimepiride, glibenclamide, glipizide, and gliclazide are widely used now. These medications are considerably more powerful, reasonably priced, and have beneficial effect on HbA1c (Erdin *et al.*, 2023). Sulfonylureas can be used with other oral antidiabetics and insulin but must be done with precaution due to the risk of hypoglycaemia (DeFronzo *et al.*, 2019).

Meglitinide

Meglitinides or Glinides, are insulin secretagogues that cause pancreatic β -cells to depolarise, which in turn increases the release of insulin. Repaglinide and Nateglinide are examples of glinides (Lv *et al.*, 2020). Renal failure patients can also utilise repaglinide. Patients with a glomerular filtration rate of 20–40 ml/min can get it in low doses (Perkovic *et al.*, 2019). Glinides and sulfonylureas have different

structures, they both act on different β -cell receptors to increase insulin production. Sulfonylureas, however, have a shorter duration of action than Glinides (Giaccari *et al.*, 2021). Glinides are rapid-acting prandial glucose regulators and have the ability to effectively reduce post-prandial hyperglycaemia when taken with meals (Levetan, 2007). Repaglinide and nateglinide both decreased HbA1c levels in individuals with T2D, according to a Cochrane analysis of 15 studies that evaluated the effects of glinides (Black *et al.*, 2007). HbA1c decreased by 0.1% to 2.1% with repaglinide and by 0.2% to 0.6% with nateglinide. The efficacy of Repaglinide in reducing HbA1c levels was comparable to that of metformin (Black *et al.*, 2007). Compared to metformin monotherapy, combination treatment with glinides may enhance glycaemic control (Yin *et al.*, 2014). Hypoglycaemia is among the main issues surrounding the usage of glinides. However, this mild to moderate in most cases and don't call for help. Nevertheless, glucose monitoring is crucial from the start of treatment until safety is confirmed (Guardado-Mendoza *et al.*, 2013).

α -glucosidase inhibitors

α -glucosidase inhibitors function by delaying the small intestine's breakdown and absorption of carbohydrate, which suppresses the rise in blood glucose levels following meals. They inhibit carbohydrate absorption by blocking the breakdown of polysaccharides into monosaccharides by digestive enzymes in the small intestine (Akmal *et al.*, 2024). There are three common types of α -glucosidase inhibitors namely, acarbose, miglitol, and voglibose. These inhibitors can be derived from medicinal plants, microbes (including bacteria and fungi), or synthesized chemically (Chougale *et al.*, 2009). They are weakly effective but can provide glycaemic control without weight gain. Also, they do not cause hypoglycaemia and may prevent the progression of diabetes in prediabetics. Adverse consequences may include bloating, diarrhoea, abdominal pain, rare hepatotoxicity, and iron deficiency (Dash *et al.*, 2018). Although they do not cause hypoglycaemia in monotherapy, they may lead to hypoglycaemia when used in combination with sulfonylurea, glinide, or insulin. The main contraindications of α -glucosidase inhibitors include conditions such as malabsorption, partial dehydration, inflammatory bowel illness, and chronic ulceration intestinal obstruction, cirrhosis, pregnancy, and lactation (Hossain *et al.*, 2020).

Insulin and incretin mimetics

Incretin-effective drugs and dipeptidyl peptidase 4 inhibitors

Incretins are metabolic hormones secreted postprandially by L-cells of the small intestine to enhance insulin secretion from pancreatic β -cells (Hansen *et al.*, 2010). These hormones are secreted after a meal intake. Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are

two key incretin hormones promotes the production of insulin and prevent the release of glucagon, which helps to reduce blood sugar levels (Saini *et al.*, 2023). These hormones can be degraded by an enzyme called dipeptidyl peptidase 4 (DPP4). DPP4 inhibitors work by inhibiting the activity of the DPP4 enzyme, thereby prolonging the action of GLP-1 and GIP (Gilbert *et al.*, 2020). Consequently, insulin secretion is sustained to lower blood sugar levels and reduces micro and macrovascular activities (Gilbert *et al.*, 2020; Saini *et al.*, 2023). DPP4 inhibitors belonging to the gliptins class increase insulin secretion in a glucose-dependent manner. Sitagliptin, vildagliptin, saxagliptin, linagliptin, and alogliptin are drugs in this group. They decrease glucagon secretion. These medications are typically ingested orally and can be utilized either on their own or in conjunction with other antidiabetic drugs. DPP4 inhibitors are generally well-tolerated, have few side effects and can reduce HbA1c by 0.6-1.1% (Vaughn *et al.*, 2010; Mackin *et al.*, 2022). However, treatment costs are high. In combination with insulin, DPP4 inhibitors may contribute to a reduction in insulin requirement, which could indirectly contribute to weight loss. Also, they can be used in combination with metformin in elderly patients to reduce the risk of hypoglycaemia. DPP4 inhibitors are known to increase islet cell function by increasing pancreatic α -cells and β -cells function (Drucker *et al.*, 2006). They do not increase the risk of cardiovascular disease (Deacon, 2020). The possible side effects of DPP4 inhibitors are headache, nasopharyngitis, upper respiratory tract infection, mild gastrointestinal discomforts acute pancreatitis, urinary tract infections, hepatic dysfunction, inflammatory bowel diseases, skin reactions, arthralgia, and myalgia (Saini *et al.*, 2023).

Sodium glucose co-transporter-2 (SGLT-2) inhibitors

Another class of the new-generation oral antidiabetic medications is the class referred to as the sodium-glucose co-transporter-2 (SGLT-2) inhibitors. By preventing glucose reabsorption in the proximal renal tubules, they reduce blood glucose levels in an insulin-independent manner (Davies *et al.*, 2022). They can be used in all phases of diabetes since they don't require insulin to function (Heerspink *et al.*, 2020; Mende *et al.*, 2022; Davies *et al.*, 2022). Due to their ability to eliminate excess glucose and slow down the course of kidney damage in diabetic patients, SGLT-2 inhibitors are associated with weight reduction, which is one of their main benefits (Ding *et al.*, 2022). SGLT-2 inhibitors cause the kidneys to eliminate glucose, which lowers calories and causes weight loss to occur. The body may remove 60–100 g of glucose per day in a glucose-dependent manner (Pereira *et al.*, 2019). SGLT-2 inhibitors may decrease the risk of heart failure and cardiovascular events (Belancic *et al.*, 2023). SGLT-2 inhibitors may also exert anti-hypertensive effects and contribute to cardiovascular protection by a variety of mechanisms, including lowering uric acid levels, reducing body weight, diuresis and natriuresis, ketogenesis, etc. (Kario *et al.*, 2020). Moreover, these medications can lower diastolic and systolic blood pressures by 1.46 mmHg 2.46 mmHg, respectively (Mazidi *et al.*, 2017). An example of an SGLT-2

inhibitor is Bexagliflozin, a highly specific and potent SGLT-2 inhibitor (Zhang *et al.*, 2011; Azzam *et al.*, 2021). The most common adverse reactions of SGLT-2 inhibitors are genitourinary infections and ketoacidosis, as well as off-target adverse responses, such as fracture, lower limb amputation risk, and tumours (Ni *et al.*, 2020).

Amylin analogues

Amylin is a hormone that delays the emptying of the stomach and suppresses glucagon secretion and food intake, thereby controlling the rate of glucose release into the circulation (Padhi *et al.*, 2020). Amylin analogues are a class of chemical analogues developed to mimic the action of human amylin since the hormone is deficient in diabetic patients (Bower *et al.*, 2016). Pramlintide is a synthetic analogue of amylin that is not associated with any major long-term side effects but shows mild to moderate adverse reactions including nausea and vomiting, headache, anorexia, reduced appetite, and hypoglycaemia (Ryan *et al.*, 2009, Hieronymus & Griffin, 2015).

Although there have been advancements in pharmacological therapies for the management of diabetes and its associated issues over the past decade, high morbidity and mortality rates are still being reported. Synthetic medications are the commonly used therapeutic approaches but come with high costs and detrimental side effects (Chaudhury *et al.*, 2017). In South Africa, the low-to-middle-class population bears this burden, since they frequently find it difficult to pay for high-quality healthcare. (Mayosi *et al.*, 2009; Mutyambizi *et al.*, 2020). Moreover, the ability of these synthetic drugs to suppress oxidative stress and mitigate its influence in diabetic complications may not be as potent as natural and/or dietary antioxidants like polyphenols. Hence, both the high- and medium- to low-income population are beginning to explore fruits, vegetables, and traditional plant-based remedies to prevent and manage long-term, non-communicable illnesses including diabetes and heart problems due to their rich antioxidant constituents (Mothibe *et al.*, 2019).

Natural antioxidants found in fruits can reduce oxidative stress and slow the development of associated metabolic and cardiovascular diseases (Wallace *et al.*, 2020). According to recent studies, non-edible wastes from many fruits, such as fruit peels, seeds, husks, etc. contain valuable nutrients and bioactive substances, such as polyphenols, carotenoids, dietary fibres, vitamins, peptides (Aqilah *et al.*, 2023). In some instances, they contain a higher concentration of some known bioactive phenolics than the corresponding edible portions of the fruits (Rafiq *et al.*, 2018; Esparza *et al.*, 2020), which could be of medicinal relevance if they are explored as sources of therapeutic agents (Chukwuma, 2024).

2.9 Fruit wastes as potential sources of bioactive phytochemicals

Fruit and vegetable companies produce significant amounts of waste, such as peels (25-30%), seeds, skins, shells, pods, cores, pulp, pomace, etc. (Rifina *et al.*, 2021). Fruit waste can be turned into high-value commodities through recycling and resource recovery, in line with the "circular economy" concept. Resource recovery is essential to effectively explore the medicinal properties of fruit waste. **Figure 2.6** depicts the recovery process of the bioactive compounds in fruit wastes and their health-related applications.

Research has demonstrated that a considerable amount of essential nutrients and phytochemicals are present in the peels, seeds, and other parts of fruits that are usually discarded. Bioactive substances such as vitamin C with phytochemical substances such as carotenoids, phenolic acids, flavonoids, and anthocyanins (Barros *et al.*, 2019; Kurek *et al.*, 2019). The documented pharmacological potentials of fruit wastes include antioxidant, anti-diabetic, tissue-protective, anti-hypertensive, anti-dyslipidaemia, anti-obesogenic anti-cancer properties, etc., which are highlighted in **Table 2.2**.

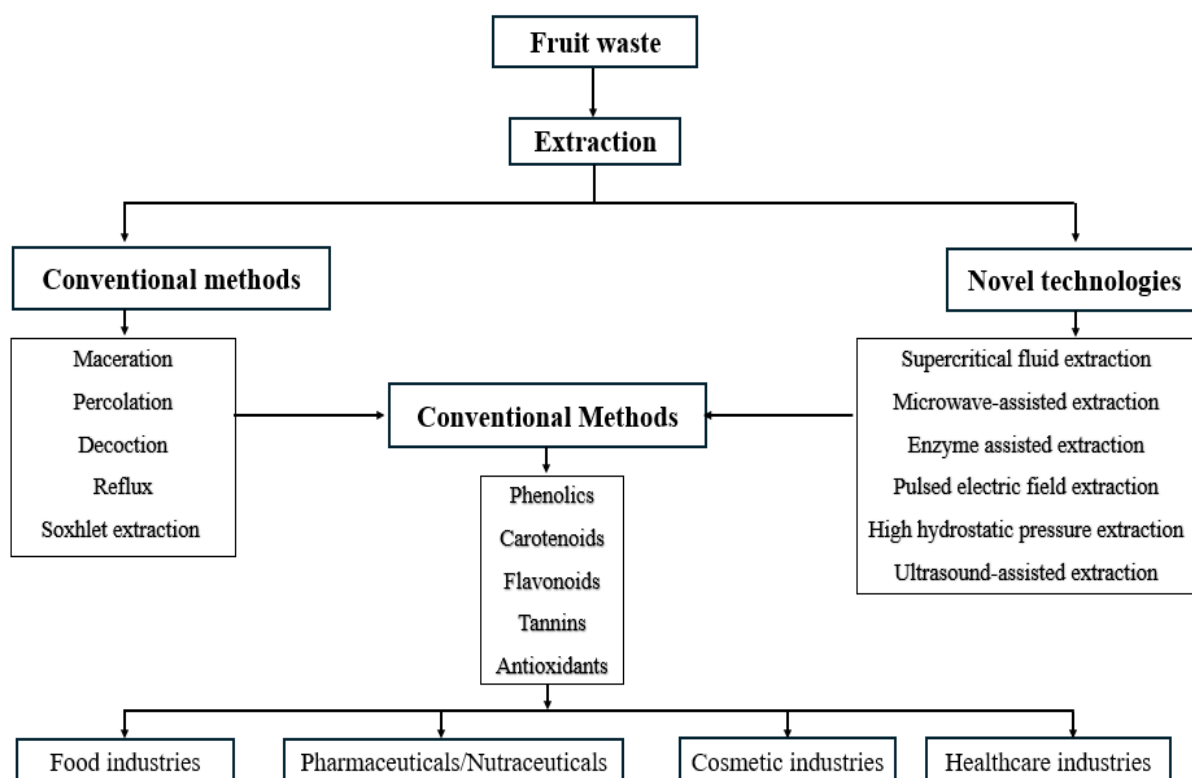


Figure 2.6: Recovery and applications of bioactive chemicals from fruit wastes (Nirmal *et al.*, 2023).

Table 2.2: *In vitro* antioxidant and glycaemic control potential and phytochemical profile of wastes from fruits.

Fruit scientific name (common name)	Taxonomic family	Parts used	Pharmacological action	Phytochemical profile of extract (Method of analysis)	References
<i>Prunus domestica</i> L. (European plum)	Rosaceae	Pomace and peel	• Fe³⁺ reducing antioxidant activity • ABTS radical scavenging activity • DPPH radical scavenging activity	Neochlorogenic acid, chlorogenic acid, 4- <i>p</i> -coumaroylquinic acid, quercetin-3- <i>O</i> -rutinoside, isorhamnetin-3- <i>O</i> -glucoside, kaempferol derivatives, cyanidin-3- <i>O</i> -glucoside, cyanidin-3- <i>O</i> -rutinoside and peonidin-3- <i>O</i> -rutinoside (LC-PDA-MS)	Michalska <i>et al.</i> , 2019, Dulf <i>et al.</i> , 2016
<i>Punica granatum</i> L. (Pomegranate)	Lythraceae	Peel	• Fe³⁺ reducing antioxidant activity • NO radical scavenging activity • α-Amylase inhibitory activity • DPPH radical scavenging activity • Inhibited glucose-induced protein glycation • Modulated glucose uptake or utilization in	Gallic acid, vanillic acid, caffeic acid, epicatechin, vanillin, ferulic acid, and quercetin (HPLC)	Chukwuma <i>et al.</i> , 2020

Chang liver
cells

<i>Prunus salicina</i> Lindl. (Japanese plum)	Rosaceae	Peel	• Oxygen and DPPH radicals scavenging activity	Cyanidin, neochlorogenic acid, rutinoid and quercetin (HPLC-UV)	de Beer <i>et al.</i> , 2012
<i>Citrullus lanatus</i> (Thunb.) Matsum. & Nakai (Watermelon)	Cucurbitaceae	Peel	• Fe³⁺ reducing antioxidant activities • ABTS radical scavenging activity • α-Amylase and α-glucosidase inhibitory activity • DPPH radical scavenging activity	Dihydroferulic acid 4-sulfate, isolariciresinol 9-O-beta-D-glucoside, citric acid, gingerol and curcumenol (LC-QTOF-MS) Citrulline, flavonoids, saponin, tannins and alkaloids	Akshaya <i>et al.</i> , 2018, Jibril <i>et al.</i> , 2019, Francis <i>et al.</i> , 2018
<i>Ananas comosus</i> L. (Pineapple)	Bromeliaceae	Peel	• DPPH radical scavenging activity • Inhibited NO activity • α-Glucosidase inhibitory activity	Ferulic acid, gallic acid, epicatechin, syringic acid, phenylacetic acid, malonic acid, succinic acid, glyceric acid, glucaric acid (1H NMR spectra)	Azizan <i>et al.</i> , 2020, Erukainure <i>et al.</i> , 2011
<i>Malus domestica</i> (Suckow) Borkh. (Apple)	Rosaceae	Seed and peel	• ABTS and DPPH radicals scavenging activity	Chlorogenic acid, p-coumaric acid, (+) catechin, (-)epicatechin, procyanidin B2, procyanidin B1, procyanidin C1, phloretin	Duda-Chodak <i>et al.</i> , 2011; Henríquez <i>et al.</i> , 2010

			• F^{3+} reducing antioxidant power	xyloglucoside, phloridzin, quercetin glycosides, cyanidin-3-galactoside (HPLC)	
<i>Mangifera Indica</i> L. (Mango)	Anacardiaceae	Peel	• α-Amylase and α-glucosidase inhibitory activity • Scavenged OH^- and Inhibit O_2^-	Gallic acid, protocatechuic acid, chlorogenic acid, ferulic acid (LC-MS)	Gondi <i>et al.</i> , 2015, Jiang <i>et al.</i> , 2010
<i>Carica papaya</i> L. (Pawpaw)	Caricaceae	Peel and seed	• DPPH and ABTS radical scavenging activity • Fe^{2+} and $FeCl_3$ reducing antioxidant activity		Ang <i>et al.</i> , 2012; Egbunu, 2017
<i>Vitis vinifera</i> L. (Grapes)	Vitaceae	Seed	• Inhibited both salivary and pancreatic α-amylases	• Epigallocatechin, epicatechin gallate, catechin-phloroglucinol, epicatechin-phloroglucinol, epigallocatechin-phloroglucinol (Reversed-phase HPLC) • Petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside, peonidin-3-O-acetylglucoside, malvidin-3-O-acetylglucoside (HPLC/DAD-MS analysis)	Fernández and Labra, 2013; Kato-Schwartz <i>et al.</i> , 2020; Kadouh <i>et al.</i> , 2016

			Gallic acid, caffeic acid, p-coumaric acid, rutin, quercetin 3-O-glucoside, myricetin, resveratrol, kaempferol (HPLC)		
<i>Citrus sinensis</i> (L.) Osbeck (Grapefruit)	Rutaceae	Peel	- F³⁺ reducing antioxidant potentials - α-Amylase and α-glucosidase inhibitory activity - ABTS and DPPH radicals scavenging activity	Isonaringin, hesperidin, neohesperidin, naringenin, hesperetin, isosinensetin, nobiletin, tangeretin (HPLC-DAD-ESI-MS)	Oboh <i>et al.</i> , 2010; Alrasheid <i>et al.</i> , 2019; Castro-Vazquez <i>et al.</i> , 2016
<i>Prunus persica</i> (L.) Siebold & Zucc. 1845 (Peaches)	Rosaceae	Peel	- α-Glucosidase inhibitory activity - F³⁺ reducing antioxidant power - DPPH and ABTS radicals scavenging activity	Procyanidin C1, Procyanidin B1, Epicatechin-epicatechin-epicatechin, procyanidin dimer, procyanidin trimer isomer 2, procyanidin trimer isomer 1, 8,8'-methylenebiscatechin, procyanidin B2, catechin, phloridzin, naringenin, neochlorogenic acid, 3-O-feruloylquinic acid, caffeoylquinic acid, chlorogenic acid (UPLC-Q-TOF/MS)	Zhang <i>et al.</i> , 2020, Suleria <i>et al.</i> , 2020
<i>Litchi Chinensis</i> Sonn. (Litchi)	Sapindaceae	Seed and peel	α-Amylase and α-glucosidase inhibitory activity	Procyanidin A1, A2, B1 and B2, epicatechin, quercetin, rutin, phlorizin, cinnamtannin B1 and B2,	Li <i>et al.</i> , 2012; Jiang <i>et al.</i> , 2013;

			• ABTS and DPPH radicals scavenging activity	aesculitannin C and cyanidin-3-glucoside, epicatechin gallate, epigallocatechin gallate	Yao <i>et al.</i> , 2021
<i>Persea Americana</i> Mill. (Avocado)	Luraceae	Peel and seed	• α-Amylase and α-glucosidase inhibitory activity • Nitric oxide (NO) radical scavenging ability • ABTS radicals scavenging activity	Syringic acid, eugenol, vanillic acid, guaiacol, kaempferol, catechin, p-hydroxybenzoic acid, ferulic acid, apigenin, naringenin, epigallocatechin, epicatechin, luteol (GC)	Oboh <i>et al.</i> , 2013; Oboh <i>et al.</i> , 2014

Polyphenols are naturally occurring organic compounds composed of phenol units. They are abundant in plants and, thus are important secondary metabolites of plants with a broad range of biological effects (Prabhu *et al.*, 2021). The major classes of polyphenols include flavonoids, polyphenolic amides, lignans, stilbenes, and phenolic acid. The flavonoids include flavonols, isoflavones, flavanones, anthocyanins, and proanthocyanins (Mehra *et al.*, 2020; Baniwal *et al.*, 2021). Clinical trial data indicate the efficacy of polyphenols in the pretreatment and prevention of a wide range of chronic illnesses, such as cancer, type 2 diabetes, degenerative diseases, anti-inflammatory, antioxidative, cardiovascular, immune-related, osteoporosis, and other neurodegenerative diseases (Behl *et al.*, 2020). They assist in the secretion of insulin, which helps maintain the blood glucose level and increase glucose tolerance and insulin sensitivity (Behl *et al.*, 2020). The consumption of a polyphenol-rich diet could reduce LDL-cholesterol, and total cholesterol levels, which ultimately control the blood pressure level (Tresserra-Rimbau *et al.*, 2017).

Flavonoids are a main group of the plant-based metabolites of polyphenolic compounds. The structural composition of flavonoids is 15- carbon atoms, comprising of 2 aromatic rings linked by a 3-carbon-atom chain (Harborne, 2013). Flavonoids can be divided into several categories depending upon the C ring to which the B ring is attached, its configuration, and oxidation of the C ring. The different categories of flavonoids include flavones (chrysin, apigenin, baicalein), flavonols (quercetin, kaempferol), isoflavones (daidzein, glycitein), flavan-3-ols (gallocatechin, catechin, epicatechin), flavanones (hesperetin, naringenin) and anthocyanidins (delphinidin, peonidin, cyanidin, pelargonidin)

(Rasouli *et al.*, 2017; Singla *et al.*, 2019). Flavonols and flavones are the largest subgroups of polyphenols among all flavonoids and are the main components of flavonoids (Zhang *et al.*, 2021). Flavonoids are powerful antioxidant that can improve the body's antioxidant status due to their free radical scavenging properties (Brglez *et al.*, 2016). More evidence has shown that flavonoids may also potentiate anti-inflammatory effect, prevent and control obesity, and improve hepatic lipid metabolism (Wen *et al.*, 2021). For this study, we shall be exploring the medicinal phytochemistry of peel from avocado (*Persea americana* Mill.) and litchi (*Litchi chinensis* Sonn.) fruits due to the documented ethnomedicinal and diabetes and oxidative stress-related pharmacological properties of the wastes from these fruits (Obboh *et al.*, 2016; Araujo *et al.*, 2021; Chukwuma *et al.*, 2021).

2.10 Avocado (*Persea americana* Mill.)

Avocado (*Persea americana* Mill.) is an evergreen tree species and the most economically important species of the *Lauraceae* family (Araújo *et al.*, 2018). The avocado fruit is a berry with a single large seed and a highly variable species (Cowan *et al.*, 2016) (**Figure 2.7**). It is classified within the genus *Persea* and subgenus *Persea*. The fruit is widely cultivated in tropical and subtropical climates worldwide and consists of numerous hybrids or varieties, including the Hass, Fuerte, Reed, and Colinred, varieties. The Hass and Fuerte varieties are among the most consumed varieties (Rodríguez-Carpena *et al.*, 2011; Bost *et al.*, 2011). The avocado fruit is well-known for its high nutritional value, unique taste, and healthy oil, which has been associated with the compounds present in the lipid fraction, such as omega fatty acids, phytosterols, tocopherols, and squalene (Maz *et al.*, 2014).

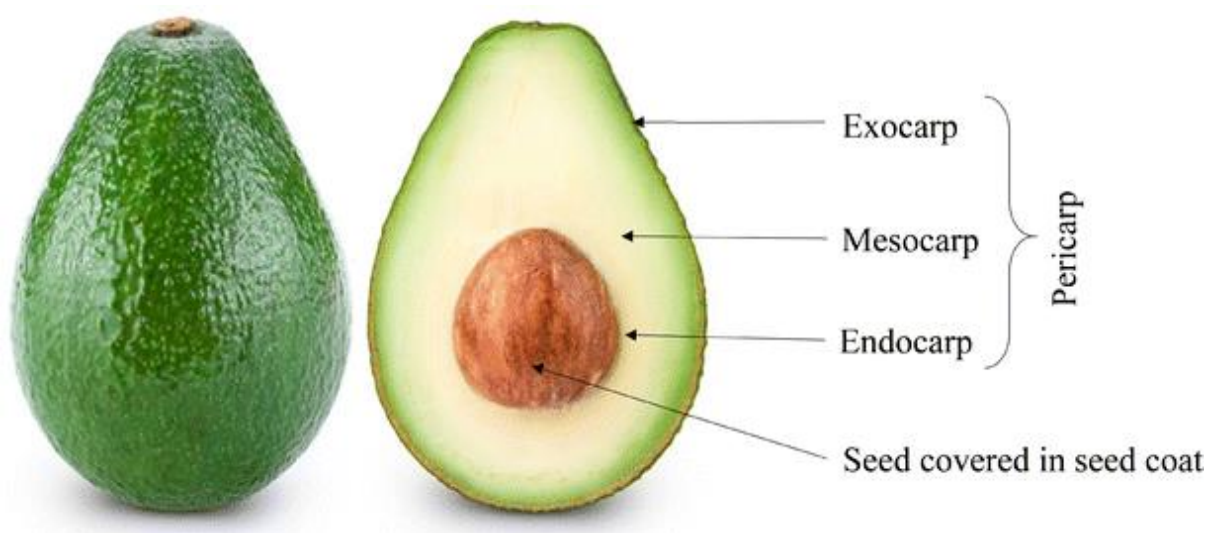


Figure 2.7: Avocado fruit and its different parts (Magwaza *et al.*, 2015).

Avocado contains polyphenolic compounds that have antioxidant effects and thus could prevent or delay the development of degenerative diseases (Alkhalaf *et al.*, 2019). Sugars present in avocados, such as

mannoheptulose and perseitol have been linked to the production of antimicrobial peptides, such as human β -defensin 2 (HBD-2), and the modulation of the proinflammatory response induced by lipopolysaccharide (LPS). It has also been reported that these sugars regulate the invasion of the yeast *Malassezia furfur* in keratinocyte cultures (human epidermal cells) (Donnarumma *et al.*, 2007). Avocados are notable for their potassium content (>500 mg/100 g of fresh weight), potassium intake helps to maintain cardiovascular health and myocardial function by regulating blood pressure. In addition, potassium regulates the electrolyte balance in the body, which is important for the conduction of electrical signals in the heart (Dreher *et al.*, 2013). The high potassium and low sodium contents in the diet are shown to protect against cardiovascular diseases (Cowan *et al.*, 2016). Nonetheless, phenolic compounds and other antioxidant molecules have also been reported in avocado fruit, which influences the antioxidant benefits of the fruit.

2.10.1 Antioxidant and phytochemical profile of avocado fruit

The Hass cultivar is the most explored avocado variety in terms of its antioxidant properties, which can perhaps be attributed to the popularity and easier availability of this variety. It is evident from the studies performed so far that phenolic compounds (including phenolic and hydroxycinnamic acids, flavonoids, and condensed tannins), carotenoids, α , β , γ , and δ -tocopherols, acetogenins, monounsaturated and polyunsaturated fatty acids are some of the antioxidants found in avocado (Boyadzhieva *et al.*, 2018a; Boyadzhieva *et al.*, 2018b; Lyu *et al.*, 2023). Moreover, most of these studies have reported significant positive correlations between the phenolic compounds and the antioxidant capacity of avocado extracts (Boyadzhieva *et al.*, 2018a; Boyadzhieva *et al.*, 2018b). Phenolic compounds found in avocados were shown to reduce oxidation, inflammation, and platelet aggregation (Dreher *et al.*, 2013). Several studies have reported that different parts of the avocado fruit contain potent phenolic antioxidants such as chlorogenic-, quinic-, succinic-, pantothenic-, abscisic-, ferulic-, gallic-, sinapinic-, *p*-coumaric-, gentisic-, protocatechuic-, 4-hydroxybenzoic-, and benzoic- acids, quercetin, quercetin-3-glucoside, quercetin-3-rhamnoside, vanillin, *p*-coumaroyl-D-glucose, catechins, (-)-epicatechin, and procyanid (Stefano *et al.*, 2017; Tremocoldi *et al.*, 2018, Santana *et al.*, 2019;). **Table 2.3** presents the antioxidant and phytochemical constituents of avocado fruit.

Table 2.3: *In vitro* antioxidant and phytochemical constituents of avocado fruit.

Part Studied	Types of Extract	Pharmacological action	Antioxidant constituents	References
Pulp	Expeller pressed oils	• ABTS radical scavenging activity		Santana <i>et al.</i> , 2019

Seed and seed coat	Accelerated solvent extraction	• ABTS and DPPH radical scavenging activity • Oxygen radical absorbance capacity	Condensed tannins, phenolic acids, and flavonoids	Figueroa <i>et al.</i> , 2018
Seed	Methanol and 50% (v/v) ethanol	• ABTS and DPPH radical scavenging activity • Oxygen radical absorbance capacity • Methoxy radical scavenging activity • Fe³⁺ reducing antioxidant activity	Chlorogenic acid, (–)-epicatechin, catechins and procyanidins	Segovia <i>et al.</i> , 2018
Seed	Aqueous extract	• Oxygen radical absorbance capacity	Epicatechin and chlorogenic acid	Calderón-Oliver <i>et al.</i> , 2016
Pulp and seed	Different solvents for different assays	• DPPH radical scavenging activity	Carotenoids, phenolic compounds, flavonoids, vitamin C, and tocopheryl acetate	Vinha <i>et al.</i> , 2013
Pulp	Aqueous and ethanolic	• DPPH radical scavenging activity • Fe³⁺ reducing antioxidant activity	Positive correlations between FRAP and total phenolics, DPPH, and total phenolics	Wang <i>et al.</i> , 2012
Pulp	Hydrophilic and lipophilic extracts	• ABTS and DPPH radical scavenging activity • Oxygen radical absorbance capacity	A positive correlation was observed between radical scavenging and palmitoleic, oleic, linoleic, and α -linolenic acids.	Villa-Rodríguez <i>et al.</i> , 2011

Pulp	Tetrahydrofuran	• DPPH radical scavenging activity	A slight positive correlation against stearic acid content.	Plaza <i>et al.</i> , 2009
Pulp and seed	Freeze-dried samples	• Fe ³⁺ reducing antioxidant activity	Vitamin C, anthocyanin, and C7 sugars.	Bertling <i>et al.</i> , 2007
Seed	80% (v/v) ethanol with ultrasonic extraction	• ABTS and DPPH radical scavenging activity • Fe ³⁺ reducing antioxidant activity	Procyanidin B2 and epicatechin, trans-5-O-caffeoyl-D-quinic acid, procyanidin B1 and catechin	Tremocoldi <i>et al.</i> , 2018
Pulp and seed	Ethyl acetate, 70% (v/v) acetone, and 70% (v/v) methanol	• ABTS and DPPH radical scavenging activity • Cupric ion reducing antioxidant capacity	Catechins, procyanidins, hydroxycinnamic acids and hydroxybenzoic acids	Rodriguez-Carpena <i>et al.</i> , 2011
Seed	80% (v/v) methanol	• ABTS and DPPH radical scavenging activity • Oxygen radical absorbance capacity	Flavanol monomers, proanthocyanidins, hydroxycinnamic acids and flavonol glycosides	Kosińska <i>et al.</i> , 2012

Adopted from Bhuyan, *et al.*, 2019

Furthermore, reported studies suggest that avocado fruit may have glycaemic control, anti-obesogenic, and blood lipid regulatory potentials. Avocado phytosterols were found to inhibit cholesterol absorption and synthesis by mimicking its molecular structure, which resulted in lowered total cholesterol levels in the body (Pieterse *et al.*, 2005). In a randomized controlled trial, obese patients consuming low-, moderate- and high-fat diet meals containing avocado fruit showed decreased total and LDL cholesterol levels (Wang *et al.*, 2015). It has also been documented that including avocado in meals could reduce the risk of vascular damage and heart disease (Fulgoni *et al.*, 2013). A large amount of indigestible carbohydrates have been found in avocados, which are linked to blood glucose and cholesterol regulation (Pahua-Ramos *et al.*, 2012). Avocado oil has been shown to have a positive effect on metabolic syndrome. In fructose-fed rats, It reduced inflammatory events and exhibited positive results in the biochemical indicators, including reducing triglycerides and LDL levels (Carvajal-Zarrabal *et al.*, 2014).

While avocado fruit may possess metabolic and antioxidant medicinal benefits, documented evidence has shown the peel also contains bioactive and antioxidant compounds, which could be of medicinal relevance. The peel contains secondary metabolites like flavonoids, tannins, and anthocyanins, which influence its documented antioxidative, antimicrobial, anticancer, anti-hypertensive, and anti-inflammatory properties (Morais *et al.*, 2015; Katja *et al.*, 2019; Araujo *et al.*, 2021).

2.10.2 Antioxidant potential and phytochemical profile of Avocado fruit peel

Avocado peel accounts for around 18% of the total fresh weight of the fruit (Figueroa *et al.*, 2018; Tremocoldi *et al.*, 2018). Phenolics present in the peel have been linked with multiple health benefits, such as antitumor, anti-aging, antidiabetic, antioxidant, and anti-inflammatory properties (Ahangarpour *et al.*, 2019; Saavedra *et al.*, 2017). It has been documented that the peel of avocado possesses higher total phenolic content, radical scavenging capacity, and antioxidant activity than the edible pulp, suggesting that the peel could be promising source of antioxidant phenolics (Figueroa *et al.*, 2018). More than 30 distinct phenolic compounds and polymeric compounds (hydroxycinnamic acids, flavonols, and flavan-3-ols) are present in avocado peels. Quinic acid, a notable hydroxycinnamic acid found in avocado peels, which are precursors of 5-O-caffeoylquinic acid or chlorogenic acid (Akan, 2021), a phenolic acid with anti-hyperglycaemic property and a remarkable DPPH radical scavenger capacity (Karasawa and Mohan, 2018; Ahangarpour *et al.*, 2019). Quercetin derivatives make up the majority of the flavonols found in avocado peels according to studies by Lopez-Cobo *et al.* (2016) and Melgar *et al.* (2018). The peel has also been shown to contain A-type procyanidins and flavan-3-ols (epi)catechin derivatives, which are important factors influencing the peel's antioxidant qualities (Tremocoldi *et al.*, 2018; Rodríguez-Martínez *et al.*, 2021; Lyu *et al.*, 2023). Several studies have consistently reported the antioxidant potential of avocado peel, which was stronger than that of the seed and the pulp (Rodríguez-Martínez *et al.*, 2021; Rahman *et al.*, 2022; Ferreira *et al.*, 2022; Lyu *et al.*, 2023). The recovery of bioactive products from avocado peel may lead to the discovery of therapeutic agents, which can foster its therapeutic applications (Kosińska *et al.*, 2012).

2.11 Litchi (*Litchi chinensis* Sonn.)

Litchi chinensis Sonn. (also known as Chinese cherry, lychee nut, leechy, Lichee, Litchi, Lychee, Mountain Lychee, and Water Lychee) is a fruit tree belonging to the Sapindaceae family (Queiroz *et al.*, 2018). It is a tropical and subtropical fruit with a delicious taste and sweet odour of rose. It has an oval shape with a rough pink to red leathery pericarp that surrounds a juicy and fleshy white/translucent aril (**Figure 2.8**). A smooth brown seed is embedded in the fleshy edible aril. Litchi fruit has a high commercial value because of its good nutritional value and global consumption. It is present in many

countries of Southeast Asia, the Indian subcontinent, Southern Africa, and other tropical or subtropical areas (Queiroz *et al.*, 2018). Litchi is found to possess a myriad of bioactive compounds in its seed, pulp, and pericarp (Ibrahim *et al.*, 2015). Fresh litchi pulp is mostly consumed as a fruit processed into various products, such as canned litchi, litchi wine, litchi beverage (litchi juice), dried litchi, frozen litchi, litchi honey, litchi jelly, and so forth (Sarkar *et al.*, 2018). Litchi contains various bioactive ingredients, including flavonoids, terpenoids, phenolics, saponins, oils, organic acids, fatty acids, amino acids, and minerals, with anti-tumour, hypoglycaemic, antioxidant, and other pharmacological properties (Yao *et al.*, 2021; Chukwuma *et al.*, 2021).

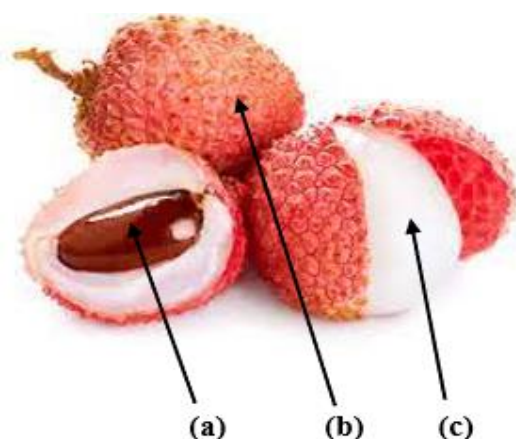


Figure 2.8: Litchi fruit showing the (a) seed, (b) pericarp/peel, and (c) edible pulp (Chukwuma *et al.*, 2021).

2.11.1 The bioactive potential of litchi fruit in metabolic and oxidative diseases

Documented evidence has proven that the different parts of litchi fruit possess pharmacological potential that could be of medicinal relevance in metabolic and oxidative health. The seed water extract exerted hypoglycaemic effect in T2D rats by improving insulin resistance, while concomitantly suppressing lipid peroxidation and improving lipid profile (Guo *et al.*, 2004). Furthermore, an important oligomeric polyphenol (Oligonol) from litchi has been reported to possess anti-obesogenic potential in both experimental animals and humans (Sakurai *et al.*, 2013). Documented reports suggest that litchi seed may exert anti-obesogenic effects through several mechanisms, including reducing visceral adipose tissue deposition, regulating adipogenesis and lipid accumulation, improving insulin sensitivity in adipose tissue, and inhibiting the action of pancreatic lipase (Queiroz *et al.*, 2018; de Almeida *et al.*, 2018; Qi *et al.*, 2015; Mhatre *et al.*, 2019; Xiang *et al.*, 2022). Studies in 3T3-L1 pre-adipocytes showed that litchi seed water extract decreased adipogenesis by inhibiting preadipocyte differentiation, attributable to the downregulation of genes involved in adipogenesis (Qi *et al.*, 2015).

Litchi seed may also contain glycaemic control phenolics and nutrients. Hydroalcoholic extract, isolated compounds (including quercetin, pavenannin B2, procyanidin A2, and phlorhizin) and polysaccharide isolate of the seed potentially inhibited the activity of α -glucosidase (Ren *et al.*, 2011; 2013; Choi *et al.*,

2017; Wu *et al.*, 2020) and protein tyrosine phosphatase 1B (Choi *et al.*, 2017), suggesting that the seed may control postprandial blood glucose by suppressing carbohydrate digestion and glucose absorption and preserving insulin sensitivity. In high-fat-diet (HFD)/streptozotocin (STZ)-induced diabetic rats, polyphenol-rich extracts of litchi seeds alleviated diabetic alterations by reducing fasting blood and urine glucose levels, as well as the symptoms of polyuria, polydipsia, and hyperphagia (Li *et al.*, 2015; Man *et al.*, 2017). The extract treatment protected against diabetes-induced pancreatic, hepatic, and renal pathologies and improved glucose tolerance and insulin resistance by modulating the expressions of hepatic Akt, GLUT-4, and PI-3K and downregulating glucagon expression (Li *et al.*, 2015; Man *et al.*, 2017). A more recent study has shown that the pulp from the immature fruit scavenged DPPH and ABTS radicals and exerted hypoglycaemic effects in Wistar rats (Contreras-Castro *et al.*, 2022).

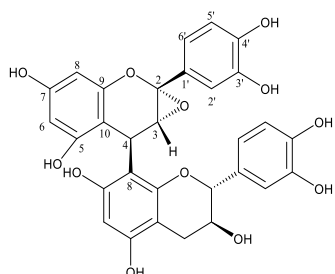
A previous review (Chukwuma *et al.*, 2021) has shown that more antidiabetic studies have been conducted on the seed of litchi fruit than the peel, warranting the need for more studies to probe the antidiabetic potential of the peel. Nonetheless, the few antidiabetic studies that have been conducted suggest that the fruit peel has glycaemic control potential.

2.11.2 The bioactive potential of litchi fruit peel in metabolic and oxidative diseases

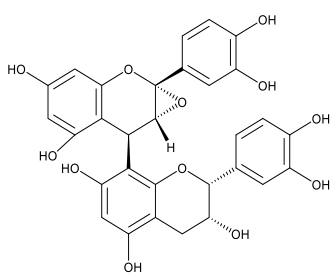
The pericarp or peel of litchi fruit is a rich source of flavonoids and anthocyanins, including procyanidin B2, B4, epicatechin, cyanidin-3-retinoside, cyanidin-3-glucoside, quercetin-3-retinoside, and quercetin 3-glucoside (**Figure 2.9**). These compounds are already established for their high free radical scavenging and glycaemic control potentials (Li *et al.*, 2007; Chukwuma *et al.*, 2021). *In vitro* study has shown that hydroalcoholic extracts of litchi peel have mild and strong inhibitory activity on α -amylase and α -glucosidase, respectively (Tanaka *et al.*, 2024), suggesting its potential to suppress postprandial glycemia.

Furthermore, the *in vivo* glycaemic control antioxidant potentials of litchi peel and its phytoconstituents have also been demonstrated. The peel flour and procyanidin-rich fraction from the peel reduced blood glucose and improved blood lipid profile in high-fat-diet-fed rats and mice (Rong *et al.*, 2017; Queiroz *et al.*, 2018). A recent study has further shown that litchi peel ethanol extract ameliorated T2D pathologies in *db/db* mice by reducing blood glucose, hepatic gluconeogenesis, oxidative stress, and associated inflammatory responses (Yang *et al.*, 2024). Oligonol is an optimized phenolic product derived from litchi peel polyphenols containing catechin-type monomers and low-molecular-weight oligomers. Administering oligonol-rich extract to diabetic rats reduced blood glucose and enhanced the secretion of insulin from β -cells (Park *et al.*, 2018). The extract reduced pancreatic oxidative stress and inflammation and improved pancreatic histology, which may partly influence its ability to preserve insulin secretion (Park *et al.*, 2018). According to the study of Park *et al.* (2018), catechin, epicatechin, epigallocatechin gallate, and procyanidins A were the major flavonoids identified in the peel's oligonol-

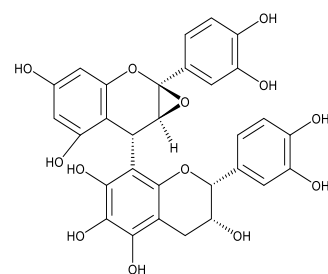
rich extract, which could be some of the bioactive compounds influencing the documented antidiabetic effects of the extract. Moreover, documented reports have shown that these flavonoids are potent metabolic, vascular, and oxidative health-promoting phenolics in tea leaves and several fruits and fruit wastes (Fu *et al.*, 2017; Rauf *et al.*, 2019).



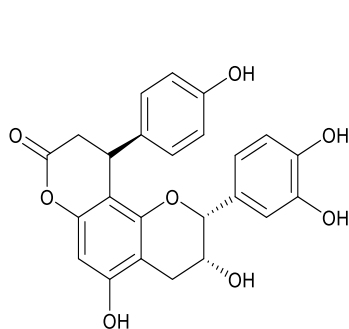
2 α ,3 α -epoxy-5,7,3',4'-
tetrahydroxyflavan-(4 β -8-
catechin)



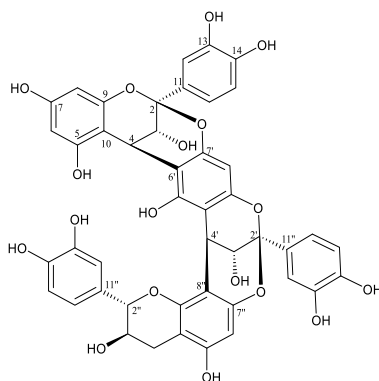
2 α ,3 α -epoxy-5,7,3',4'-
tetrahydroxyflavan-(4 β -8)-
epicatechin



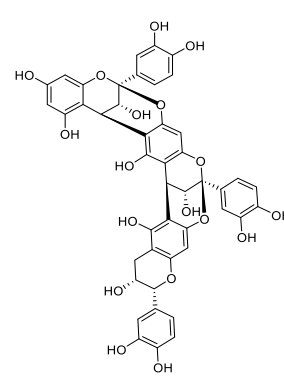
2 β ,3 β -epoxy-5,7,3',4'-
tetrahydroxyflavan-(4 α -8-
epicatechin)



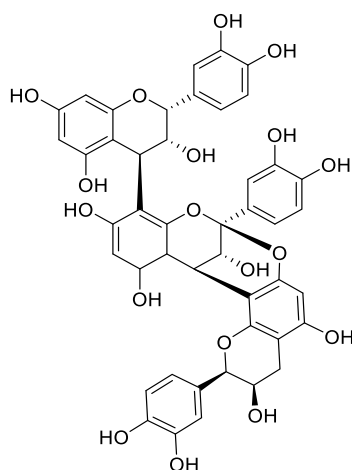
Epicatechin-(7,8-bc)-4 β -(4-
hydroxyphenyl)-dihydro-2(3H)
pyranone



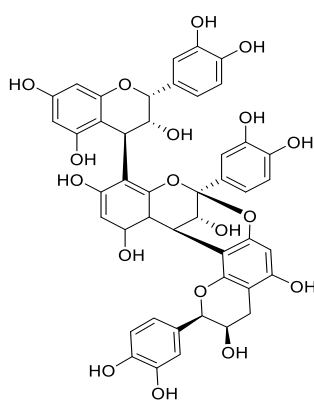
Litchitannin A1



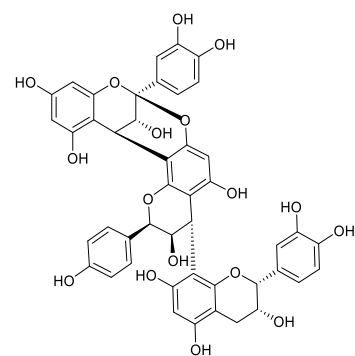
Litchitannin A2



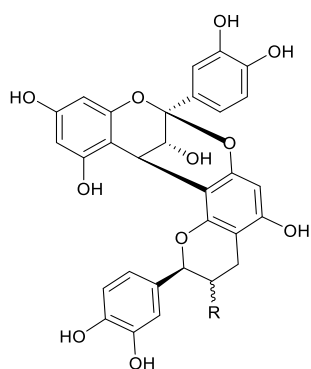
Aesculitannin A2



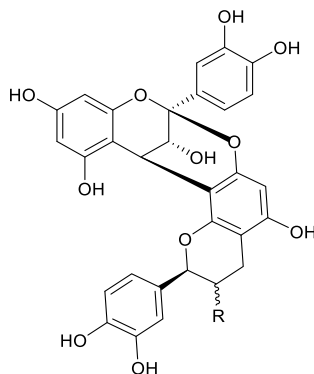
Epicatechin-(2 β →O→7,4 β →8)-
epiafzelechin-(4 α →8)-epicatechin



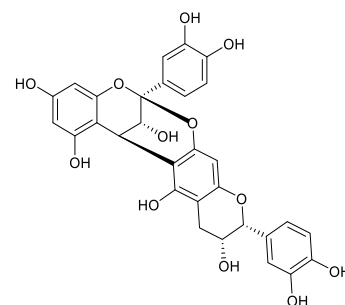
Proanthocyanidin A1



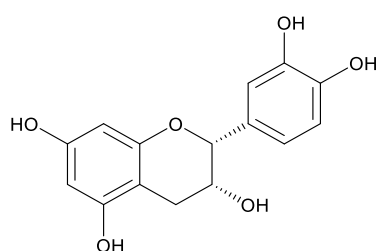
Proanthocyanidin A1



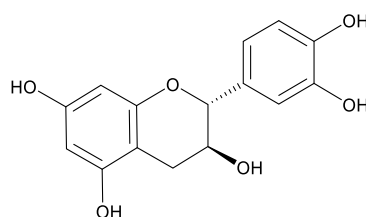
Proanthocyanidin A2



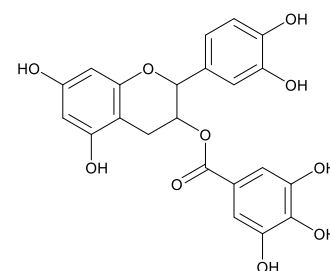
Proanthocyanidin A6



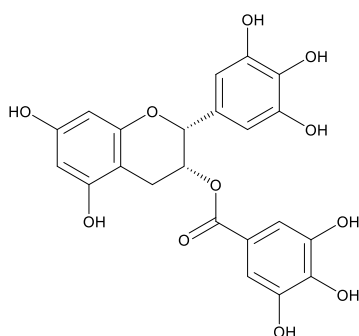
Epicatechin



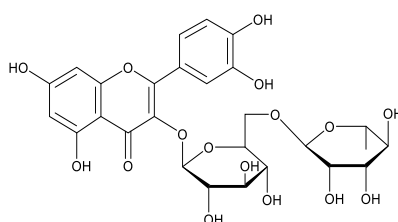
Catechin



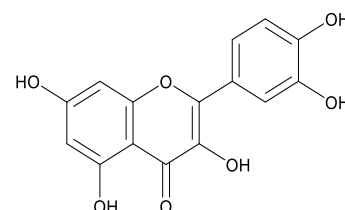
Epicatechin gallate



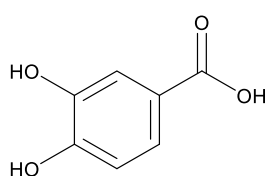
Epigallocatechin gallate



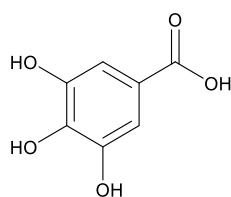
Rutin



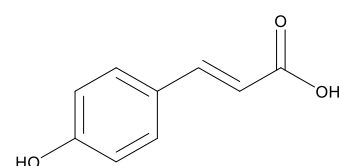
Quercetin



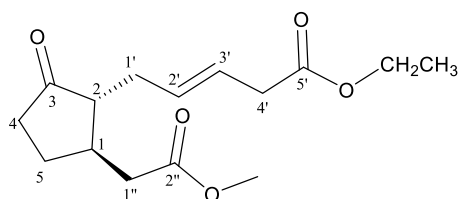
Protocatechuic acid



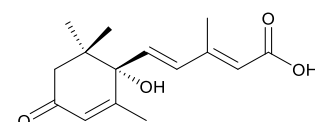
Gallic acid



p-Coumaric acid



(1R,2R,2'E)-2-(5'-ethoxy-5'-oxo-2-penten-1-yl)-3-oxo-cyclopentane
acetic acid methyl ester



(S)-(+)-trans-abscisic acid

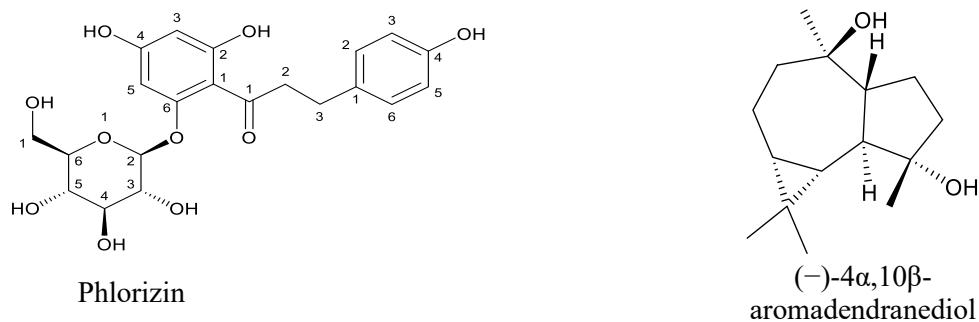


Figure 2.9: Structures of bioactive compounds in the peel and seed of litchi fruit.

Furthermore, a more recent antidiabetic study (Chauhan *et al.*, 2021) in alloxan-induced type 1 diabetic Wistar rats demonstrated the blood glucose lowering, and antioxidant effect of litchi peel extract, suggesting that litchi peel extract possess ameliorative potential against diabetes associated pathologies. **Table 2.4** summarizes the glycaemic control, antioxidant, and phytochemical profiles of the peel of litchi fruit.

Table 2.4: Glycaemic control, antioxidant, and phytochemical profiles of the peel of litchi fruit.

Extract/isolate	Experimental model and treatment	Pharmacological effects	Phytochemistry of part, extract, fraction, or isolate	Reference
Oligonol isolated from aqueous extract	10 days oral treatment (10 and 20 mg/kg bw) in STZ-induced diabetic SD rats	• Reduced serum glucose • Increased serum and pancreatic insulin • Increased serum c-peptide	Catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, procyanidin A1, A2, B1 and B2	Park et al., 2018
Flour	21 days feeding of diet containing flour (5 and 10%) in HCD-fed male Wistar rats	• Reduced blood glucose, weight gain, BMI, serum TG, TC and LDLC • Increased HDLC	Polyphenols, flavonoids and anthocyanins	Queiroz et al., 2018
Procyanidin-rich fraction from acetone extract	24 wks oral treatment (100 mg/kg bw) in HFD fed ApoE KO mice	• Reduced plasma glucose, LDLC, TC and TG • Reduced % bw of mesenteric and		Rong et al., 2018

		perirenal adipose tissues		
		• Reduced aortic lesion • Reduced aortic iNOS activity and expression and nitric oxide production • Reduced expression of NADPH oxidases (NOX-2 and NOX-4)		
Compounds isolated from 85% ethanol extract	In vitro	Scavenged DPPH radical and reduced Fe^{3+}	(+)-Isolariciresinol 9- <i>O</i> - α -l-arabinopyranoside, burselignan 9- <i>O</i> - α -l-arabinopyranoside, (-)-secoisolariciresinol and 9- <i>O</i> - α -l-arabinopyranoside,	Ma et al., 2014
Hydroalcoholic extracts (enzyme assisted extraction)	In vitro	Scavenged DPPH radical and reduced Fe^{3+}	Gallic acid, procyanidin A2 and B2, quercetin, rutin, catechin and epicatechin	Kessy et al., 2018
Polysaccharide extracted by microbial fermentation	In vitro	• Scavenged DPPH radical • Reduced oxidative DNA damage	Galactose, xylose, rhamnose and mannose	Lin et al., 2013
Procyanidins-rich fraction and sub-fraction of aqueous extract	In vitro	Scavenged DPPH radical and reduced Fe^{3+}	Catechin, proanthocyanidin B2, pavenannin B2, Litchitannin B1, dimers of	Gong et al., 2018

			proanthocyanidin A, trimers of complex proanthocyanidin A and B, trimers of complex proanthocyanidin A and B and procyanidin A2	
Methanol extract	6 wks oral treatment (20, 100 and 200 mg/kg bw) in CCl ₄ -treated male ICR mice	Increased hepatic GPx and GR activities	Epicatechin and procyanidin A2	Chen et al., 2017a
Oligonol isolated from aqueous extract	10 days oral treatment (10 and 20 mg/kg bw) in STZ-induced diabetic SD rats	• Reduced diabetes induced elevation in the protein expression levels of pancreatic inflammatory markers and associated signalling factors • Improved diabetes-induced histological changes in the pancreas • Reduced serum and pancreatic ROS production and lipid peroxidation	Catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, procyanidin A1, A2, B1 and B2	Park et al., 2018
Resin-purified ethanol extract	5 days oral treatment (50, 100, 200 mg/kg	• Reduced lipid peroxidation in myocardial tissue	procyanidin B2, (-)-epicatechin, epicatechin-(4β →	Chen et al., 2017b

bw.) in	• Increased SOD	8,2 β $\rightarrow$ O $\rightarrow$ 7)-
myocardial	and GPx activities	epicatechin-(4 β $\rightarrow$ 8)-
ischemic SD rat	in myocardial	epicatechin, A-type
model	tissue	procyanidin trimer, B- type procyanidin dimer and procyanidin A2

Abbreviations: “BMI”, body mass index; “GPx”, glutathione peroxidase; “GR”, glutathione reductase; “HDL”, high-density lipoprotein cholesterol; “HFD”, high-fat-diet; “LDL”, low-density lipoprotein cholesterol; “ROS”, reactive oxygen species; “SD”, Sprague Dawley; “SOD”, superoxide dismutase; “STZ”, streptozotocin; “TC”, total cholesterol; “TG”, triglyceride.

2.12 Problem Statement

Diabetes is a notable health problem that is causing a socioeconomic burden in South Africa due to mortality and morbidity outcomes, as well as unaffordable healthcare costs in the low to middle-income population. Most of the people in the low to middle-income population rely on medicinal plants, including fruits and vegetables, because of the minimal toxicity concerns and holistic medicinal profile of medicinal plants, which has been attributed to bioactive phenolics in them. Documented evidence has shown that most bioactive constituents of many South African fruits are also deposited more in the non-edible parts such as the peels, pomace, seeds, etc. Unfortunately, this part of fruits is constantly wasted and contributes to a notable percentage of fruit waste generated daily in South Africa. Thus, limiting the potential medicinal relevance of fruit wastes, as well as their possible bioeconomic importance. Avocado and litchi are popular in South African fruits with documented medicinal and phytochemical profiles. Several key bioactive phenolics and flavonoids have been identified in the peel of these fruits. While these phenolics and flavonoids may influence the anti-diabetic and antioxidant potentials of the peel from these fruits, their pharmacological contributions remain elusive. Studies have not adequately correlated the principal phytoconstituents in the peel of these fruits to the corresponding antioxidant and glycaemic control bioactivities, which could give more insight into the bioactive phytochemistry of peel from these fruits. It is, therefore, imperative to undertake studies that will help understand the antidiabetic and antioxidant phytochemistry of peel from these fruits, as this could encourage their effective use as a potential therapy for diabetes and associated complications.

2.13 Aim and objectives

Aim

This study was done to investigate the antidiabetic and antioxidative phytochemistry of the peel of avocado and litchi fruit.

Objectives

The following are the objectives of this study:

- Subject the peel of fruits to solvent extraction
- Conduct in vitro anti-oxidative and anti-diabetic bioassay-guided fractionation
- Isolate and spectrometrically characterize the bioactive principle
- Subject potent extract(s) to *in vivo* anti-diabetic and antioxidant assessment.

CHAPTER 3

MATERIALS AND METHODS

3.1 Litchi fruit peel and solvent extraction

Litchi (McLean's Red cultivar) and avocado (Var. Fuerte) fruits were purchased from a local fruit and vegetable store in Bloemfontein, South Africa. The fruit was washed with clean water. The peel was removed and oven-dried (Model No. 072160, Prolab instrument, Sep Sci., Johannesburg, South Africa) at 30 °C. The dried peel was pulverized. Approximately 300 g of the pulverized dried peel was defatted with 1.5 liters of hexane and then sequentially extracted using 1.5 liters of dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH), and water, in an order of increasing polarity. The extraction was done for 48 h at room temperature on an orbital shaker [OrbiShake, Model 262, Labotec 106 (Pty) Ltd, Johannesburg, South Africa] set at 125 rpm. The extraction was done twice for each solvent. The DCM, EtOAc, and MeOH extracts were concentrated using a Buchi 108 Rotavapor® R-300 [Labotec (Pty) Ltd, Johannesburg, South Africa] and dried under a fume hood. The water extract was recovered by freeze drying (Martin Christ Alpha 1-2 LDplus Freeze Dryer 110, Separations, Johannesburg, South Africa). The extracts were weighed, transferred into clean screw-cap vials, and stored at -20 °C.

3.2 Screening the phenolic, flavonoid, antioxidant and glycaemic control properties of extracts

The extracts were screened at a concentration of 55 µg/mL. The total phenol and flavonoid contents were measured using previously described methods (Chukwuma *et al.*, 2020).

3.2.1 Total phenol content estimation

Into a 96-well clear bottom plate containing 25 µL of the extracts or gallic acid (Merck, Johannesburg, South Africa) standard solutions (3.75 to 120 µg/mL final assay concentrations), 125 µL of 10x diluted Folin–Ciocalteu reagent (Merck, Johannesburg, South Africa) was aliquoted. Into the mixture, 100 µL of 0.7 M Na₂CO₃ was added. The plate was left to stand for 30 min at room temperature. Thereafter, the optical density was recorded at 765 nm using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, USA). The absorbances were blanked using those of the sample blanks or the solvent blanks. The total phenol content was computed from a gallic acid standard curve ($y = 0.0775x$; $R^2 = 0.9999$) of concentration (µg/mL) versus the corresponding absorbances at 765 nm as follows:

$$\text{Total phenol content (mg/g GAE)} = \frac{C \times SV}{M}$$

Where, “C” is the concentration (mg/mL) extrapolated from gallic acid standard curve; “SV” is the sample volume (mL), and “M” is the amount (g) of the sample in the “SV” (mL) of the sample solution; “GAE” means gallic acid equivalent.

3.2.2 Total flavonoid content estimation

Into a 96-well clear bottom plate containing 20 μL of the extracts or quercetin (Merck, Johannesburg, South Africa) standard solutions (3.75 to 120 $\mu\text{g/mL}$ final assay concentrations), 80 μL of 98% ethanol was aliquoted. Into the mixture, 100 μL of 2% AlCl_3 was added. The plate was left to stand for 1 h at room temperature. Thereafter, the optical density was recorded at 430 nm. The absorbances were blanked using those of the sample blanks or the solvent blanks. The total flavonoid content was computed from a quercetin standard curve ($y = 0.0346x$; $R^2 = 0.9969$) of concentration ($\mu\text{g/mL}$) versus the corresponding absorbances at 430 nm as follows:

$$\text{Total flavonoid content (mg/g QE)} = \frac{C \times SV}{M}$$

Where, “C” is the concentration (mg/mL) extrapolated from quercetin standard curve; “SV” is the sample volume (mL), and “M” is the amount (g) of the sample in the “SV” (mL) of the sample solution; “QE” means quercetin equivalent.

For the antioxidant screening, the Fe^{3+} reducing (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and nitric oxide (NO) radicals scavenging and anti-linoleic acid peroxidative models were used. These were performed according to the protocols reported previously (Chukwuma *et al.*, 2020; Motloung *et al.*, 2020; Mashile *et al.*, 2020)

3.2.3 Ferric (Fe^{3+}) antioxidant capacity measurement

Into a 96-well clear bottom plate containing 25 μL of the extracts or the positive controls (ascorbic acid and Trolox; Merck, Johannesburg, South Africa) or gallic acid standard solutions (3.75 to 120 $\mu\text{g/mL}$ final assay concentrations), 25 μL each of 0.2 M phosphate buffer (pH, 6.6) and 1% potassium ferricyanide solutions was added. The plate was left to stand for 20 min at 50 $^{\circ}\text{C}$. Next, 25 μL of 10% trichloroacetic acid solution, 100 μL of distilled water and 50 μL of 0.1% FeCl_3 solution were added, consecutively. Immediately, the optical density was recorded at 700 nm. The absorbances were blanked using those of the sample blanks or the solvent blanks. The Fe^{3+} reducing capacity was computed from

a gallic acid standard curve ($y = 0.037x - 0.0047$; $R^2 = 1$) of concentration ($\mu\text{g/mL}$) versus the corresponding absorbances at 700 nm as follows:

$$\text{Total phenol content (mg/g GAE)} = \frac{C \times SV}{M}$$

Where, “C” is the concentration (mg/mL) extrapolated from gallic acid standard curve; “SV” is the sample volume (mL), and “M” is the amount (g) of the sample in the “SV” (mL) of the sample solution; “GAE” means gallic acid equivalent.

3.2.4 DPPH radical scavenging capacity measurement

Into a 96-well clear bottom plate containing 75 μL of the extracts or the positive controls (ascorbic acid and Trolox) or the solvent (control), 37.5 μL of a 0.3 mM DPPH solution was added. The plate was left to stand for 30 min in the dark at temperature. Immediately, the optical density was recorded at 517 nm. The absorbances were blanked using those of the sample blanks or the solvent blanks. The radical scavenging activity was computed as follows:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3.2.5 ABTS radical scavenging capacity measurement

The ABTS radical was generated by mixing and incubating equal volumes of 7 mM ABTS and 2.4 mM potassium persulfate solutions in the dark for 12 – 16 hours at room temperature. Thereafter, the mixture was diluted 5 times with methanol. Into a 96-well clear bottom plate containing 50 μL of the extracts or the positive controls (ascorbic acid and Trolox) or the solvent (control), 125 μL of the diluted ABTS radical solution was added. The plate was left to stand for 15 min in the dark at temperature. Immediately, the optical density was recorded at 734 nm. The absorbances were blanked using those of the sample blanks or the solvent blanks. The radical scavenging activity was computed as follows:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3.2.6 Nitric oxide (NO) radical scavenging capacity measurement

Into a 96-well clear bottom plate containing 75 μL of the extracts or the positive controls (ascorbic acid and Trolox) or the solvent (control), 75 μL of sodium nitroprusside (10 mM in 50 mM of phosphate buffer, pH 7.4; Merck, Johannesburg, South Africa) was added. The plate was left to stand for 2 h in the

dark at 37 °C. Next, 75 µL of Greiss reagent (Merck, Johannesburg, South Africa) was added. Immediately, the optical density at 546 nm was used to estimate the formation of NO₂. The absorbances were blanked using those of the sample blanks or the solvent blanks. The radical scavenging activity was computed as the inhibition NO₂ formation using the following formula:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3.2.7 Measurement of linoleic acid peroxidation inhibition

Into screw-cap vials containing 30 µL of the extracts or the positive control (ascorbic acid) or distilled water (vials assigned “control” and “negative control”), 30 µL of a 50 mM linoleic acid (Merck, Johannesburg, South Africa) and 20 µL of Tris-HCl buffer (100 mM, pH 7.5) were added. Next, 20 µL of FeSO₄·7H₂O (2 mM final assay concentration; Merck, Johannesburg, South Africa) was added to the test vials and the vials assigned “negative control” to induce lipid peroxidation. An equivalent volume of distilled water was added to the vials assigned “control”. The plate was left to stand for 30 min at 37 °C. Next, 80 and 50 µL of 5.5 % acetic acid and 0.25 % thiobarbituric solutions (Merck, Johannesburg, South Africa) were added, respectively, into the vials. The content of the vials was mixed, and the vials were immersed in boiling water for 30 min. After cooling, the optical density (532 nm) of aliquots (150 µL) from each vial was measured. The absorbances were blanked using those of the sample blanks or the solvent blanks. The inhibitory activity (%) on linoleic acid peroxidation was computed as follows:

$$\text{Inhibition (\%)} = \frac{(A_{\text{Negative control}} - A_{\text{Normal control}}) - (A_{\text{Test}} - A_{\text{Normal control}})}{(A_{\text{Negative control}} - A_{\text{Normal control}})} \times \frac{100}{1}$$

Where “A” means absorbance.

3.2.8 Measurement of α-glucosidase inhibition

Into a 96-well clear bottom plate containing 25 µL of the extracts or the positive controls (acarbose; Merck, Johannesburg, South Africa) or the solvent (control), 50 µL of rice α-glucosidase (EC Number: 232-604-7; Merck, Johannesburg, South Africa) solution (4 U/mL in 100 mM phosphate buffer, pH 6.8) was added. The plate was left to stand for 10 min at 37 °C. Next, 25 µL of *p*-nitrophenyl glucopyranoside substrate (Merck, Johannesburg, South Africa) solution (10 mM in 100 mM phosphate buffer, pH 6.8) was added. The enzyme/substrate reaction was allowed to take place for 20 min at 37 °C, before stopping with 100 µL of 100 mM Na₂CO₃. Immediately, the optical density was recorded at 405 nm. The absorbances were blanked using those of the sample blanks or the solvent blanks. The inhibitory effect of the extracts or acarbose on the enzyme activity was computed as follows:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3.2.9 Measurement of α -amylase inhibition

Into a screw-cap vials containing 75 μL of the extracts or the positive controls (acarbose) or the solvent (control), 75 μL of porcine pancreatic amylase (EC Number: 232-565-6; Merck, Johannesburg, South Africa) solution (3 U/mL in 100 mM phosphate buffer, pH 6.8) was added. The plate was left to stand for 15 min at 37 $^{\circ}\text{C}$. Next, 75 μL of starch substrate solution (1% in 100 mM phosphate buffer, pH 6.8) was added. The enzyme/substrate reaction was allowed to take place for 30 min at 37 $^{\circ}\text{C}$, before adding 75 μL of dinitrosalicylate color reagent (Merck, Johannesburg, South Africa). The content of the vials was mixed, and the vials were immersed in boiling water for 10 min. After cooling, the absorbance of aliquots (150 μL) from each vial was measured at 540 nm. The absorbances were blanked using those of the sample blanks or the solvent blanks. The inhibitory effect of the extracts or acarbose on the enzyme activity was computed as follows:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3.3 Bioassay guided fractionation of the avocado peel methanol extract

About 4.1 g of the methanol extract (without tannin) was subjected to a gradient column (silica gel) fractionation. Thin layer chromatography (TLC) was first used to determine the appropriate solvent system for the gradient column fractionation. The mobile phase started with a solvent system volume ratio of 8:1.5:0.5 (hexane, chloroform, and methanol, respectively) and ended with a volume ratio of 0:9:1 (hexane, chloroform, and methanol, respectively). Collected fractions with fairly similar TLC profiles were pooled together into nine different groups labelled as fractions 2 to 10. The fractions were recovered by air drying.

3.3.1 Qualitative phytochemical profiling and antioxidant evaluation of fractions from the avocado peel methanol extract

Total phenol and flavonoid content, FRAP, and DPPH and ABTS radical scavenging activities were measured for all the 9 fractions (fractions 2 to 10) and compared to positive controls (ascorbic acid and Trolox) using the protocols described in **sub-section 3.2**. For all the above-mentioned assays, the fractions and positive controls were tested at a concentration of 25 $\mu\text{g/mL}$. Fraction 8 (a pool of fractions with similar TLC profile), obtained from a mobile phase solvent system volume ratio of 3:6.5:0.5

(hexane, chloroform, and methanol, respectively), had the highest total phenol content and the most potent FRAP and radical scavenging activity compared to the other fractions. Thus, fraction 8 was subjected to LC-MS analysis.

3.3.2 Liquid chromatography-mass spectroscopic (LC-MS) analysis of fraction 8 from avocado peel methanol extract

LC-MS analysis was performed by the Central Analytical Facility, Stellenbosch University, Western Cape. A Waters Synapt G2 (Waters Corporation, Milford, MA, USA), ESI probe, and ESI Pos, at a 15 V cone voltage. The operation of liquid chromatography was done using an Acquity binary solvent manager. The chromatographic column used was a HSS T3 (Waters Corporation, Milford, MA, USA). It had a dimension of 2.1×150 mm and a particle size of $1.8 \mu\text{m}$. Two solvent systems (A and B) were used as the mobile phase. Solvent system A was water containing 0.1% methanoic acid, while solvent system B was acetonitrile containing 0.1% methanoic acid. A 0.25 mL/min flow rate was applied for the gradient chromatographic separation at 30°C . The gradient condition followed the following sequence: 100% (A):0% (B), 10 min; 72% (A):28% (B), 21 min; 60% (A):40% (B), 50 s; 0% (A):100% (B), 2 min. The extract was dissolved in a mixture of acetonitrile and water (1:1 v/v), passed through a $0.22 \mu\text{m}$ filter, and injected at a volume of $20 \mu\text{L}$. The signals of the separated compounds were recorded at 254 nm. Using the accurate masses, the compounds in the fraction were tentatively determined and semi-quantified (mg/L) relative to rutin standard, and according to the extracted ions.

LC-MS showed the predominant presence of chlorogenic acid (103.5 mg/mL) and 1-*O*-caffeoylquinic acid (102.3 mg/mL) in fraction 8. Hence, it was assessed for dose-dependent antioxidant evaluation and compared to that of commercial chlorogenic acid (Merck, Johannesburg, South Africa). Also, Density Functional Theory (DFT) analysis was used to investigate the possible underlying radical scavenging mechanisms of chlorogenic acid.

3.3.3 Dose-dependent radical scavenging and anti-linoleic acid peroxidative assessment of fraction 8 and chlorogenic acid

The dose-dependent DPPH radical scavenging and anti-linoleic acid peroxidative activities of fraction 8 and chlorogenic acid were measured at using the protocol reported in **sub-sections 3.2.4 and 3.2.6**. The radical scavenging activity of the samples were measured at concentrations of 3.75, 7.5, 15, 30, and $60 \mu\text{g/mL}$, while the anti-linoleic acid peroxidative activity of the samples were measured at concentrations of 5, 10, 20, 40, and $80 \mu\text{g/mL}$.

3.3.4 Measurement of the antioxidant capacity of fraction 8 and chlorogenic acid in Chang cells

The effect of the tested samples (fraction 8 and chlorogenic acid) and ascorbic acid (positive control) on lipid peroxidation and the activity of key antioxidant enzymes was measured in Chang cells (ATCC® CCL-13™) induced with oxidative stress. The detailed protocol for this assay has recently been reported (Motlounge *et al.*, 2020). In summary, oxidative stress was induced in cultured cells using FeSO₄ (1 mM in assay volume) after pre-treatment with the different concentrations (10, 20, 40, and 80 µg/mL in assay volume) of the fraction, chlorogenic acid and ascorbic acid. The thiobarbituric acid (TBA) method was used to measure the level of lipid peroxidation in the cell lysate, which was extrapolated from an MDA standard plot as TBA reactive substances (TBARS). Also, the activities of catalase and superoxide dismutase (SOD) were measured in the lysate of cells treated with the highest concentration (80 µg/mL) of the samples using the following assay kits purchased from Merck, Johannesburg, South Africa: Catalase Assay Kit (catalog number: CAT100) and SOD Assay Kit (product number: 19160). The inhibitory potential of the tested samples on lipid peroxidation was computed using the formula below:

$$\text{Inhibition (\%)} = \frac{(T_{\text{Negative control}} - T_{\text{Normal control}}) - (T_{\text{Test}} - T_{\text{Normal control}})}{(T_{\text{Negative control}} - T_{\text{Normal control}})} \times 100$$

Where “T” is the level of lipid peroxidation in the form of TBARS; the “Normal control” denotes cells treated with the sample solvent/vehicle, without FeSO₄; the “Negative control” denotes cells treated with the sample solvent/vehicle and FeSO₄; and the “Test” denotes cells treated with the sample and FeSO₄.

3.3.5 Measurement of the inhibitory effect of fraction 8 and chlorogenic acid on ROS and NO production inhibition in RAW 264.7 cells

The effect of the tested samples (fraction 8 and chlorogenic acid) and ascorbic acid (positive control) on ROS and NO production was measured in RAW 264.7 macrophages [American Type Culture Collection (ATCC) Rockville, MD, USA; ATCC No.: TIB-71™] treated with lipopolysaccharide (LPS) (Merck, Johannesburg, South Africa). For ROS and NO production assays, the methods reported by Marrazzo *et al.* (2018) and Njoya *et al.* (2023) were adopted, respectively, with slight modifications.

RAW 264.7 macrophages were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Separations, Johannesburg, South Africa) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a CO₂ incubator (EC 160, Nüve; Separations, Johannesburg, South Africa) under standard cell culture conditions (37 °C in a humidified environment with 95% air and 5% CO₂). At about 80% confluence, the cells were trypsinized with 0.25% trypsin/EDTA (Cytiva Hyclone, USA) and split at a ratio of 1:5 for further passaging. Next, the cytotoxic effect of the tested samples on the

RAW 264.7 macrophages was measured at concentrations of (6.25–100 µg/mL) using standard MTT assay protocol.

For the ROS and NO production inhibition assay, confluent RAW 264.7 macrophages were seeded in black/clear-bottom 96-well plate at a density of 10,000 cells per well and incubated for 24 h. The cells were then pre-treated for 2 h with the tested samples. For the ROS production inhibition assay, the cells were treated at sample concentrations of 25, 50, and 100 µg/mL in incubation volume. For the NO production inhibition assay the cells were treated at sample concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL in incubation volume. Some wells were assigned the control and negative control groups, which contained cells treated with the sample solvents/vehicle ($\leq 0.5\%$ DMSO). Next, LPS (200 ng/mL in final incubation volume) was added to the wells containing the samples and the wells assigned as the negative control group, while an equivalent volume of the 0.5% DMSO was added to the wells assigned as the control group. The plates were further incubated for 24 h.

To analyze the cells for ROS production, the cells were washed with phosphate buffered saline (PBS). Next, 100 µL of fresh FBS-free culture medium, containing a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Merck, Johannesburg, South Africa) fluorescent probe (10 µM in incubation volume) was added into each well with minimal exposure to light, and the plates were further incubated for 30 min. Thereafter, the media containing the fluorescent probe was replaced with PBS, and fluorescence was measured at 485 nm (excitation) and 535 nm (emission) using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, USA). Also, cell images were obtained using a Leica DM IL LED inverted fluorescent microscope (Separations, Johannesburg, South Africa). The inhibitory potential of the tested samples on ROS production was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(F_{\text{Negative control}} - F_{\text{Normal control}}) - (F_{\text{Test}} - F_{\text{Normal control}})}{(F_{\text{Negative control}} - F_{\text{Normal control}})} \times 100$$

where “F” is the fluorescence intensity.

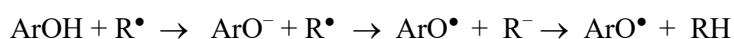
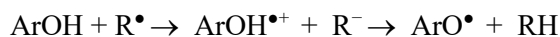
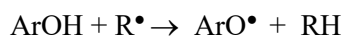
To analyze the cells for NO production post-LPS treatment, 100 µL of the incubation medium from each well of the 96-well microtitre plates was aliquoted into designated wells of a new 96-well transparent plate. An equal volume of Griess reagent (Merck, South Africa) was added to the wells and the plate was kept in the dark at room temperature for 15 min. Absorbance values were measured at 540 nm. The concentration of (µM) nitrite was calculated from a sodium nitrite standard linear plot ($y = 0.0029x + 0.0114$). The inhibitory potential of the tested samples on NO production was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(C_{\text{Negative control}} - C_{\text{Normal control}}) - (C_{\text{Test}} - C_{\text{Normal control}})}{(C_{\text{Negative control}} - C_{\text{Normal control}})} \times$$

Where “C” is the nitrite concentration (µM) extrapolated from the sodium nitrite standard plot.

3.3.6 Density functional theory (DFT) analysis

The hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET-PT), and sequential proton loss followed by electron transfer (SPLET) mechanisms were used to computationally study the DPPH radical scavenging activity of chlorogenic acid. The equations below sequentially denote the mechanisms for HAT, SET-PT and SPLET, respectively.



Where $\text{ArO}^\bullet$, ArO^- , $\text{ArOH}^{\bullet+}$, R^- and RH denote phenolic radical, phenolic anion, phenolic cation, anion radical and neutral molecule, respectively.

Geometry optimization of all the reaction species was performed using B3LYP together with the 6-311++g (d,p) basis set. This computational method is largely used for radical scavenging mechanisms and has been shown to accurately estimate the geometries and thermodynamic parameters of the reaction species (Galano and Alvarez-Idaboy, 2013). The bond dissociation enthalpy (BDE), adiabatic ionization potential (AIP), proton dissociation enthalpy (PDE), proton affinity (PA) and electron transfer enthalpy (ETE) (in kcal/mol) of the reaction species for the capture of the DPPH radical by chlorogenic acid (5-CQA) were computed for a methanol solvent-based reaction, which are parameters that inform the above-mentioned radical reaction mechanisms.

3.4 Bioassay-guided fractionation of litchi peel ethyl acetate extract and isolation/purification of epicatechin

The litchi peel ethyl acetate extract was, first, chemically profiled using LC-MS according to the protocol reported in **sub-section 3.3.2**. Using the accurate masses and extracted ions, the compounds in the fraction were tentatively determined and semi-quantified (mg/L) relative to catechin calibration and according to the extracted ions. Thereafter, the extract was subjected to gradient column fractionation. A flow diagram of the fractionation and isolation protocol is presented in **Figure 3.1**. The extract was subjected to a silica gel 60 (0.040-0.063 mm; Merck, Johannesburg, South Africa) gradient column chromatography, starting with a solvent system (DCM:MeOH: water; 9:1:0.125 v/v/v), which was pre-determined by thin layer chromatography (TLC). The column was run with decreasing volume of DCM, increasing volume of MeOH and water as shown in the Supplementary material (**Table S1 of Appendix**). The fractions obtained were pooled into 8 main fractions (**Table S1 of Appendix**) based on their TCL profiles. The fractions were identified as fractions 1 to 8.

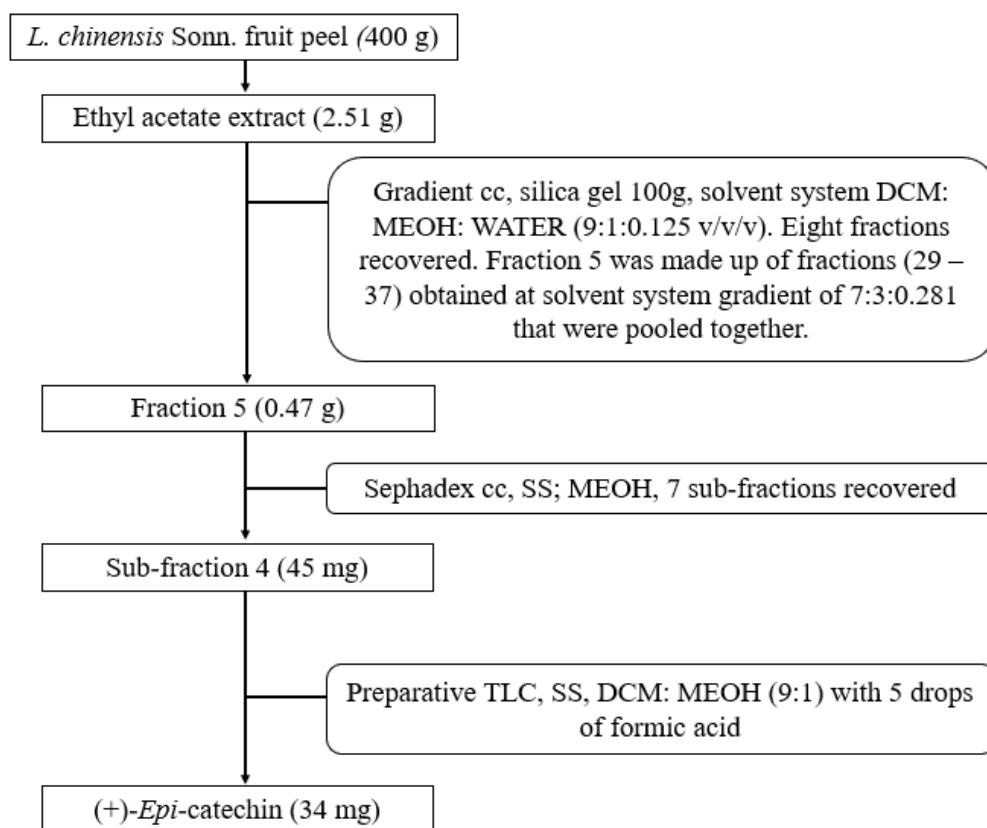


Figure 3.1: Schematic representation of the isolation of compounds from the Ethyl Acetate extract of Litchie Peel

The total phenol and flavonoid contents, as well as the antioxidant (FRAP, DPPH, and ABTS radicals scavenging activity) and glycaemic control (α -glucosidase and α -amylase inhibition) potentials of the fractions, were measured at a concentration of 35 $\mu\text{g/mL}$ according to the protocols described in **sub-section 3.2**.

Based on the bioactivity results obtained, fraction 5, which comprises a pool of fractions (29 - 37) obtained from a solvent system gradient of 7:3:0.281 v/v/v (DCM:MeOH:water), was selected for further fractionation using a Sephadex (Merck, Johannesburg, South Africa) column with 100% methanol as the solvent system. Also, LC-MS was used to chemically profile fraction 5 according to the protocol reported in **sub-section 3.3.2**. Using the accurate masses and extracted ions, the compounds in the fraction were tentatively determined and semi-quantified (mg/L) relative to catechin calibration and according to the extracted ions.

Seven sub-fractions were obtained from fraction 5 after separation in a Sephadex column. The in vitro radical (DPPH and ABTS) scavenging, anti-linoleic acid peroxidative and α -glucosidase inhibitory activities of the sub-fractions were measured at 20 $\mu\text{g/mL}$ using the protocol reported in **sub-section 3.2**. Also, the cellular glucose uptake modulatory activity of the sub-fractions was measured as described below.

3.4.1 Measurement of glucose modulatory activity in Chang cells

Chang cells were used to measure the cellular glucose uptake modulatory activity of the sub-fractions, and a previous protocol (Motlounge *et al.*, 2020) was adopted after some modifications. Chang cells (ATCC® CCL-13™; American Type Culture Collection Rockville, MD, USA) were cultured in a CO₂ incubator using DMEM-high glucose media supplemented with 10% FBS (Separations, Johannesburg, South Africa). At confluence, the cells were seeded into a 48-well plate at a density of 15,000 cells per well. After 24 h of incubation, the spent media was replaced with 350 µL of fresh media containing the sub-fractions (20 µg/mL in incubation medium). After 24 h of incubation, the media was aspirated, and the cells were washed with PBS. Next, 350 µL of RPMI media (diluted to 8 mM glucose concentration) containing the sub-fractions (20 µg/mL in incubation medium) was added to the wells. Designated wells were used as the media (containing media without cells) and control (containing media with untreated cells) groups. The plate was incubated for 3 h. Thereafter, 20 µL aliquot from each well was diluted 24 times with distilled water, and glucose concentration (in mg/mL) was measured in the diluted aliquots against a glucose standard curve ($y = 9.766x + 0.0585$) using a Glucose (GO) Assay Kit (Merck, Johannesburg, South Africa), which was then converted to concentration in millimolar (mM). The glucose uptake activity (%) of the control and test groups was computed relative to the media group using the following formula:

$$\text{Glucose uptake activity (\%)} = \frac{GC_{\text{media}} - GC_{\text{control or test}}}{GC_{\text{media}}} \times 100$$

Where “GC” is the glucose concentration in millimolar (mM).

The glucose uptake modulatory activity of the sub-fractions was computed relative to the control by subtracting the glucose uptake activity of the control from that of each fraction.

Based on the bioactivity data obtained from the sub-fractions, sub-fraction 4 was further subjected to preparative TLC (20 × 20 cm glass-backed, 0.25 mm silica plates) for the isolation of bioactive compound(s). The solvent system used was DCM: MEOH (9:1) with 5 drops of formic acid. The UV-active substances were scraped off, washed with acetone and methanol, consecutively, and recovered by filtration.

3.4.2 Nuclear magnetic resonance spectroscopy and mass spectrometry of the isolated compound

The proton (¹H) and (¹³C) NMR spectra were acquired using a Bruker Avance 400 MHz spectrometer. Deuterated DMSO solvent was used, and the working temperature was 25 °C. For ¹H resonances (0 ppm), tetramethylsilane (TMS) was employed as an internal standard. The standard pulse sequences

and processing macros given in the original program were used for advanced and two-dimensional spectra such as ^{13}C APT/DEPT and 2D COSY, NOESY, HSQC, and HMBC.

The compound(s) were subjected to low-resolution mass spectrometry. The MS experiment used an MD Sciex 3200 QTrap equipped with an electrospray (Turbo-ion spray) ionization source.

The isolated compound was assessed for dose-dependent antioxidant and glycaemic control potential using the following experimental models.

3.4.3 Measuring the inhibitory effect of the isolated compound on lipopolysaccharide (LPS)-induced ROS production in RAW 264.7 cells

The inhibitory potential of the isolated compound on LPS-induced ROS production was measured in RAW 264.7 cells at different concentrations (8.75, 17.5, 35, 70 and 140 μM) using the protocol described in **sub-section 3.2.5**.

3.4.4 Measuring the dose-dependent α -glucosidase inhibitory and glucose uptake modulatory effect of the isolated compound

The dose-dependent α -glucosidase inhibitory and glucose uptake modulatory effect of the isolated compound was measured according to protocols described in **sub-sections 3.2.8** and **3.4.1**. The dose-dependent α -glucosidase inhibitory effect of the samples and acarbose was measured at concentrations of 3.75, 7.5, 15, 30 and 60 μM . The dose-dependent glucose uptake modulatory effect of the sample was measured at concentrations of 10, 20, 40 and 80 μM and compared to that of a 2 μM metformin.

3.5 IC_{50} and EC_{50} computation

The IC_{50} and EC_{50} values of the tested samples were computed on GraphPad Prism 7 and/or MS excel 2016 software using the non-linear or linear plot of transformed ($\log 10$) sample concentrations (x-axis) versus the corresponding inhibitory bioactivities.

3.6 *In vivo* antidiabetic and antioxidant assessment of litchi peel ethyl acetate extract

The antidiabetic and antioxidant potential of the litchi peel ethyl acetate extract was investigated in a fructose/streptozotocin (STZ) model of diabetic rats.

3.6.1 Animals

The approval for the use of animals in this study was obtained from the University of Free State Animal Research Ethics Committee (EREC) on 07 February 2023 (Protocol reference number: UFS-AED2022/0028/23). Thirty-five 7 to 8 weeks old Sprague-Dawley (SD) rats were procured by the University of the Free State Animal Research Unit, where the animal experiment was conducted. The animals were allowed to acclimatize for 5 days. Thereafter, the animals were assigned into five groups containing seven animals each. The groups were named the normal control (NC), diabetic control (DBC), low-dose peel extract (LDPE), high-dose peel extract (HDPE) and metformin (MET) groups. The animals were housed two to three animals per cage in large-sized cages with the following dimensions: 598mm (L) x 380mm (W) x 200mm (H) with Grid top hatch access of 220mm (L) x 225mm (W) x 70mm (H). The animals were housed in a temperature (20 – 24 °C; set point at 22 °C) and humidity (40-70%; set point 60%) controlled room with a 12 h light-dark cycle.

3.6.2 Diabetes induction

A previous study (Wilson and Islam, 2012) has shown that the combination of 10% fructose and low dose (40 mg/kg bw) STZ could be used to develop a diabetic SD rat model that exhibits two key pathologies of T2D, namely insulin resistance and β -cell damage. This approach was adopted in inducing diabetes. First, the rats in the diabetic groups (DBC, LDPE, HDPE and MET groups) were fed *ad libitum* with 10% fructose solution in place of drinking water for 2 weeks. Thereafter, the rats were injected with a single intraperitoneal (i.p.) injection of STZ (40 mg/kg bw) dissolved in citrate buffer (pH, 4.4), while the rats in the NC group were injected with equivalent volume of citrate buffer. One week after STZ injection, the tail-tip blood glucose (mmol/L) of rats were measured using a Contour Plus Blood Glucose Monitoring System (Ascensia Diabetes Care Holdings AG, Basel, Switzerland). A multiplication factor of 18.018 was used to convert the blood glucose measurements from mmol/L to mg/dL. Animals having blood glucose values ranging from 250 to 600 mg/dL were considered diabetic and included in the study. Of the 28 rats induced with diabetes, four rats were extremely diabetic (blood glucose > 600 mg/dL), and thus were excluded from the study. Therefore, each of the diabetic groups were left with six animals each. The number of animals in the NC group was also reduced to six to be consistent with other groups.

3.6.3 Treatment

After diabetes confirmation, the rats in the different groups were administered via oral gavage with the following treatments:

- NC group: The vehicle of the extract or metformin (5% DMSO solution)
- DBC group: The vehicle of the extract or metformin (5% DMSO solution)
- LDPE: 150 mg/kg bw of extract dissolved in 5% DMSO solution
- HDPE: 300 mg/kg bw of extract dissolved in 5% DMSO solution
- MET: 200 mg/kg bw of Metformin hydrochloride (Merck, Johannesburg South Africa) dissolved in dissolved in 5% DMSO solution.

The animals were treated for 20 days during a 4-weeks intervention period. This implies that the animals were treated during the first five days in each week of the intervention period.

3.6.4 Measurement of fluid intake, food intake and non-fasting blood glucose

During the intervention period, the average daily fluid intake and average weekly food intake of the animals were measured. The tail-tip non-fasting blood glucose (NFBG) level of the animals was measured in the 6th day of the 1st, 2nd and 4th weeks of the intervention period. The NFBG was measured using a glucometer.

3.6.5 Measurement of oral glucose tolerance

The oral glucose tolerance test (OGTT) was performed on the 6th day in the 3rd week of the intervention period. The rats were fasted for 7 h (from 3:00 am to 10:00 am). Thereafter, the tail-tip blood glucose of the animals was measured (0 min), before orally administering 2 g/kg bw of glucose. The tail-tip blood glucose of the animals was then measured for 120 min at 30 min intervals. Thereafter, the animals were allowed access to food. The area under the curve (AUC) for the OGTT was calculated according to a previous report (Sakaguchi *et al.*, 2015):

$$\text{AUC (mg. h/dL)} = \frac{\text{BG0} + (\text{BG30} \times 2) + (\text{BG60} \times 3) + (\text{BG120} \times 2)}{4}$$

Where BG0, BG30, BG60 and BG120 mean blood glucose (mg/dL) at 0, 30, 60 and 120 min, respectively.

3.6.6 Euthanization and sampling

After the intervention period, the animals were euthanized using isoflurane. The blood of the animals was collected by cardiac puncture and stored in tubes without an anti-coagulant. Also, the liver, pancreas and muscle tissues of the animals were collected. The collected tissue samples were immediately frozen in liquid and stored at -80 °C. A portion of the pancreatic tissues was fixed in 10% neutral buffered

formalin (Merck, Johannesburg, South Africa) for histological assessment. The blood was centrifuged at 3500 x g for 10 min at 4 °C using a NUVE NF 800R centrifuge (Separations, Johannesburg, South Africa) and the supernatant (serum) was stored at -80 °C.

3.6.7 Measurement of liver and muscle glycogen content

The phenol–sulfuric acid method (Lo *et al.*, 1970) was used according to a modified version (Matowane *et al.*, 2023). About 0.5 g of tissue was disintegrated in a vial by adding 1.5 mL of 30% KOH solution and boiling for 40 min to form a homogenous solution. After cooling, 2 mL of 95% ethanol was used to precipitate the sugars in the homogenized tissues. This was done at 2 – 8 °C for 30 min. The supernatant was recovered and removed by centrifuging at 1200 x g for 30 min at ambient temperature. The residue or sediment was washed twice with 95% ethanol and dissolved in 3 mL of distilled water. From the dissolved residue or glycogen standards (0.005 – 0.1 mg/200 µL) 200 µL was transferred into glass vials. Then, 1 mL of phenol (5% solution) and 5 mL of H₂SO₄ (98% solution) were added, successively. The vials were gently agitated to mix. The absorbance of aliquots (250 µL) from each vial was measured at 490 nm (SpectraMax M2 microplate reader, Molecular Devices, San Jose, CA, USA). From the glycogen standard curve ($y = 0.021x - 0.0165$; $R^2 = 0.9984$), the glycogen content (mg/g tissue) of the tissues was calculated as follows:

$$\text{Tissue glycogen content (mg/g tissue)} = \frac{C \times D}{M}$$

Where, “C” is the extrapolated concentration (mg/200 µL); “D” is the dilution factor = 31, which is the dilution of 200 µL of the dissolved residue in 6.2 mL of assay volume; “M” = 0.5 g, which is the mass (g) of tissue used in the assay.

3.6.8 Measurement of serum insulin

Serum insulin was measured using an enzyme-linked immunosorbent assay (ELISA) method. A Rat/Mouse Insulin ELISA Kit (Merck, Johannesburg, South Africa; product number: EZRMI) was used to perform the assay and the serum insulin concentration was expressed in ng/mL.

3.6.9 Tissue homogenization and protein estimation

The tissues were thawed and homogenized using a method reported previously (Matowane *et al.*, 2023). About 100 mg of the different tissues (liver, pancreas and muscle) was homogenized in 1 mL of ice-cold buffer (sodium phosphate buffer containing 0.5% v/v Triton X-100 and 1 mM EDTA, pH 7.5)

using a D1000 handheld homogenizer (Merck, Johannesburg, South Africa). The supernatant of the tissue homogenates was recovered at 10 400 x g for 10 min at 4 °C. Protein concentration was measured in the supernatant. The protein concentration (mg/mL) was extrapolated from a bovine serum albumin (BSA) standard curve ($y = 0.7059x + 0.1982$; $R^2 = 0.9992$) using the bicinchoninic acid (BCA) method.

3.6.10 Measurement of muscle tissue hexokinase activity

The activity of hexokinase in the muscle tissue homogenate was measured at a protein concentration of 5 mg/mL according to the protocols described in a Hexokinase Colorimetric Assay Kit (Merck, Johannesburg, South Africa; catalogue number, MAK091). Hexokinase catalyses the phosphorylation of glucose to glucose-6 phosphate. Glucose-6 phosphate is oxidized by glucose-6 phosphate dehydrogenase, during which the oxidized form of nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH. This assay estimates hexokinase activity by measuring the NADH-mediated colorimetric reduction of a chromogenic probe over a duration of 20 min. NADH production was quantified from an NADH standard curve ($y = 0.111x - 0.0147$; $R^2 = 0.998$) of NADH amounts (2.5, 5, 7.5, 10, and 12.5 nmol/reaction mixture) versus the corresponding absorbances at 450 nm. One unit of hexokinase represents the enzyme activity that will generate 1.0 μ mol of NADH from NAD^+ per minute at pH 8.0 under room temperature. The specific activity (nmol/min/mg protein) was calculated with respect to the protein concentration of the tissue homogenates as follows:

$$\text{Specific activity (nmol/min/mg protein)} = \frac{A \times DF}{RT \times SV \times PC}$$

Where, “A” = amount (nmole) of NADH generated during reaction time; “DF” = dilution factor of sample in reaction volume; “RT” = reaction time (min); “SV” = sample volume (mL); “PC” = sample protein concentration (mg/mL).

3.6.11 Measurement of muscle tissue Akt phosphorylation

The pan-Akt and phospho-Akt levels in the muscle tissue supernatant were estimated by a colorimetric ELISA method using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (Merck, Johannesburg, South Africa; catalogue number, RAB0012) and presented as the spectrometric optical densities (OD). To estimate the levels of Akt phosphorylation, the ratio between the ODs of phospho-Akt and pan-Akt was computed.

3.6.12 Measurement of lipid peroxidation

The protocol reported in a previous study (Tshane *et al.*, 2021) was slightly modified to perform this assay. Lipid peroxidation was estimated in the serum and tissue homogenates (liver and pancreas). In a vial, the tissue homogenate or malondialdehyde (MDA) standards (100 μL), 0.25% thiobarbituric acid solution (500 μL), 20% acetic acid solution (200 μL) and distilled water (200 μL) were successively added and mixed. The MDA standards were 7.5, 15, 22.5, 30, 45 and 60 μM solutions. The vials were placed in a boiling water bath for 50 min and the absorbance of aliquots (250 μL) from each vial was measured at 532 nm. Lipid peroxidation, determined as the concentration of thiobarbituric acid reactive substances (TBARS) was extrapolated from the MDA standard curve ($y = 0.0083x + 0.0062$; $R^2 = 0.999$).

3.6.13 Measurement of superoxide dismutase activity

Superoxide dismutase activity (SOD) was measured in the serum and tissue homogenates (liver and pancreas) using an SOD Assay Kit (product number, 19160; Merck, Johannesburg, South Africa). SOD catalyses the reduction of superoxide ion. Thus, the activity of SOD in the samples was determined by measuring the inhibitory action on superoxide ion-mediated colorimetric (450 nm) reduction of a water-soluble tetrazolium salt [2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] as follows:

$$\text{Inhibition (\%)} = \frac{\text{OD}_C - \text{OD}_S}{\text{OD}_C} \times 100$$

Where “OD_C” is the optical density for colorimetric (450 nm) reduction by the control (without enzyme), while “OD_S” is the optical density for colorimetric (450 nm) reduction by the samples.

The specific activity (nmol/min/mg protein) was calculated with respect to the protein concentration of the tissue homogenates as follows:

$$\text{Specific activity (\% inhibition/mg protein)} = \frac{I \times \text{DF}}{\text{SV} \times \text{PC}}$$

Where, “I” = percentage inhibition (%) by enzyme on superoxide ion-mediated colorimetric reduction of a water-soluble tetrazolium salt; “DF” = dilution factor of sample in reaction volume; “SV” = sample volume (mL); “PC” = sample protein concentration (mg/mL).

3.6.14 Measurement of catalase activity

Catalase activity was measured in the serum and tissue homogenates (liver and pancreas) using a Catalase (CAT) Activity Assay Kit (Catalogue number, E-BC-K031-S; Biocom Africa, Johannesburg, South Africa). Catalase catalyses the decomposition of H_2O_2 , a reaction that can be chromogenically (405 nm) stopped by ammonium molybdate. The activity of catalase in the samples was estimated by measuring the residual H_2O_2 after 1 min of enzyme-substrate reaction. The residual H_2O_2 was extrapolated from a H_2O_2 standard curve ($y = 0.0307x - 0.0002$; $R^2 = 0.9998$) of H_2O_2 amounts (0, 0.5, 1, 2, 3, 4 and 5 μmol) versus the corresponding absorbances at 405 nm. The specific activity (U/mg protein) was calculated with respect to the protein concentration of the tissue homogenates as shown below. One unit of catalase will decompose 1.0 micromole of H_2O_2 per minute at pH 7.0 under room temperature at a substrate concentration of 50 mM H_2O_2 .

$$\text{Specific activity (U/mg protein)} = \frac{A \times DF}{RT \times SV \times PC}$$

Where, “A” = amount (μmole) of H_2O_2 that was used up during reaction time; “DF” = dilution factor of sample in reaction volume; “RT” = reaction time (min); “SV” = sample volume (mL); “PC” = sample protein concentration (mg/mL).

3.6.15 Histopathological examination of pancreatic tissues

The fixed pancreatic tissues were dehydrated by successive changes in concentrations (50%, 70%, 90% and 100%, respectively) of ethanol. Ethanol was removed from the tissue by repeated immersions in xylene before embedding in paraffin according to the standard protocol. The embedded tissues were cut into about 4 μm sections and mounted on slides. Hematoxylin and Eosin were used to stain tissue samples to highlight important features of the tissue samples. The DPX mounting media was applied before placing a cover slip. The slides were viewed with a Leica slide scanner.

3.7 Data and statistics presentation

The data of this study were presented as the average of the replicate data ($n = 3$ for in vitro assays and $n = \text{six animals}$ for in vivo study) along with the standard deviation ($\pm$). For each parameter, statistical analysis was done to compare the data mean of the different groups. This was done using the IBM SPSS Statistics software (version 29). The one-way ANOVA and Tukey post hoc were used for computing the multiple comparison of the data averages and statistical significance was set a $p < 0.05$.

CHAPTER 4

UNRAVELLING THE INFLUENCE OF CHLOROGENIC ACID ON THE ANTIOXIDANT PHYTOCHEMISTRY OF AVOCADO (*PERSEA AMERICANA* MILL.) FRUIT PEEL

Prescript

This chapter investigated and reported the antioxidant phytochemistry of avocado (*Persea americana* Mill.) peel. The chapter has been published in **Antioxidants** as follows: ***Izu GO***, Mfotie Njoya E, Tabakam GT, Namboozee J, Otukile KP, Tsoeu SE, Fasiku VO, Adegoke AM, Erukainure OL, Mashele SS, Makhafola TJ, Sekhoacha MP, Chukwuma CI*. (2024). *Unravelling the Influence of Chlorogenic Acid on the Antioxidant Phytochemistry of Avocado (Persea americana Mill.) Fruit Peel*. *Antioxidants*, 13(4), 456. <https://doi.org/10.3390/antiox13040456>. The above-mentioned publication was adopted in writing the content of this chapter.

4.1 Abstract

Oxidative stress is pivotal in the pathology of many diseases. This study investigated the antioxidant phytochemistry of avocado (*Persea americana* Mill) peel. Different solvent extracts (dichloromethane, ethyl acetate, methanol, and water) of avocado peel were subjected to total phenol and flavonoid quantification, as well as in vitro radical scavenging and ferric reducing evaluation. The methanol extract was subjected to gradient column chromatographic fractionation. Fraction 8 (eluted with hexane:chloroform:methanol volume ratio of 3:6.5:0.5, respectively) was subjected to LC-MS analysis. It was assessed for cellular inhibition of lipid peroxidation and lipopolysaccharide (LPS)-induced ROS and NO production. The DPPH radical scavenging mechanism of chlorogenic acid was investigated using Density Functional Theory (DFT). The methanol extract and fraction 8 had the highest phenol content and radical scavenging activity. Chlorogenic acid (103.5 mg/mL) and 1-*O*-caffeoylquinic acid (102.3 mg/mL) were the most abundant phenolics in the fraction. Fraction 8 and chlorogenic acid dose-dependently inhibited in vitro ($IC_{50} = 5.73$ and $6.17 \mu\text{g/mL}$) and cellular ($IC_{50} = 15.9$ and $9.34 \mu\text{g/mL}$) FeSO_4 -induced lipid peroxidation, as well as LPS-induced ROS ($IC_{50} = 39.6$ and $28.2 \mu\text{g/mL}$) and NO ($IC_{50} = 63.5$ and $107 \mu\text{g/mL}$) production, while modulating antioxidant enzyme activity. The fraction and chlorogenic acid were not cytotoxic. DFT analysis suggest that an electron transfer, followed by proton transfer at carbons 3'OH and 4'OH positions may be the radical scavenging mechanism of chlorogenic acid. Considering this study is bioassay-guided, it is logical to conclude that chlorogenic acid strongly influences the antioxidant capacity of avocado fruit peel

Keywords: *Avocado peel; chlorogenic acid; antioxidant; chromatography.*

4.2 Introduction

Oxidative stress remains a culprit in the development and progression of many chronic diseases (Jomova *et al.*, 2023). It is caused by a disruption in the balance between the biological production of deleterious reactive oxygen species (ROS)/reactive nitrogen species (RNS) and mitigating antioxidant actions (Bardaweel *et al.*, 2018; Hameister *et al.*, 2020). ROS and RNS play important roles in redox balance and signalling, immune response, and cellular homeostasis (Bardaweel *et al.*, 2018). However, most ROS and RNS are radical species, which are very reactive (Hameister *et al.*, 2020; Mandal *et al.*, 2022). Thus, their uncontrolled production could lead to oxidative alteration or damage of important biological molecules (Jomova *et al.*, 2023; Hameister *et al.*, 2020; Mandal *et al.*, 2022). Oxygen, unlike other elements that exist as bi-atomic molecules, has better stability in the triplet state of electron spin (the state of unpaired electrons) than in the singlet state of electron spin (the state of paired electrons), which plays a major role in the reactivity of ROS (Bardaweel *et al.*, 2018).

ROS and RNS are mostly by-products of biological signalling and/or metabolism. The commonly generated ROS and RNS include hydroxyl radicals, hydrogen peroxide, nitric oxide, peroxides, peroxynitrite, singlet oxygen, and superoxides, which are common by-products of the mitochondrial electron transport, cytochrome P₄₅₀, and NADH oxidase pathways (Jha *et al.*, 2017). They have been reported to have detrimental effects on biological molecules, which have been implicated in oxidative stress-associated diseases (Mandal *et al.*, 2022; Jomova *et al.*, 2023).

Oxidative stress is a common denominator in the pathology of many chronic diseases (diabetes, cancer, cardiovascular diseases, renal disease, mental and neurological disorders, and pulmonary diseases) and aging due to the oxidative damage caused by ROS and RNS on DNA, proteins, and membrane lipids (Jomova *et al.*, 2023). Redox signalling is important for proper myocardial functioning and is regulated by several kinases. Oxidative stress can dysregulate this signalling, which can lead to cardiovascular disorders (Jomova *et al.*, 2023). Oxidative stress can cause oxidative damage to contractile proteins, which may lead to contractile dysfunctions and vascular impairments (Steinberg *et al.*, 2013). ROS may induce pro-tumorigenic signalling, which may promote cancer cell proliferation and survival (Reczek *et al.*, 2017). In a diabetic state, glycation processes can lead to advanced glycation end product (AGE) production (Perrone *et al.*, 2020). AGEs can induce oxidative stress, which induces several stress-induced transcription factors and pro-inflammatory processes. Lipid peroxidation causes oxidative modifications to biological lipids, including membrane lipids, which compromises cell membrane integrity (Jomova *et al.*, 2023). Oxidative modifications of lipids, such as apo-lipoprotein B, may lead to the formation of oxidized low-density lipoproteins, which have been implicated in the pathology of atherosclerosis (Jomova *et al.*, 2023).

The body is equipped with antioxidant mechanisms to mop deleterious ROS and mitigate the damages caused by oxidative stress (Birben *et al.*, 2012). These include antioxidant enzyme systems, such as catalase, superoxide dismutase, and glutathione-related enzyme systems, as well as endogenous antioxidant molecules that quench pro-oxidants or enable the functioning of antioxidant pathways (Birben *et al.*, 2012). Dietary or plant-derived polyphenols are known to possess antioxidant capacity, which is key for their medicinal potential in different diseases (Sun *et al.*, 2020). Many fruits and vegetables contain these antioxidant phenolics, which contribute to their health benefits (Afam *et al.*, 2021). Despite the antioxidant benefits of fruit consumption, studies have shown that the non-edible wastes of many fruits are notable sources of phenolics, which are influential in their reported medicinal properties (Chukwuma *et al.*, 2020; Hussain *et al.*, 2022; Sha *et al.*, 2023,). For instance, the seed of avocado fruit has ethnomedicinal applications in mental disorders and obesity (Oboh *et al.*, 2016), while the peel has cosmetic applications (Ferreira *et al.*, 2022).

Avocado fruit (*Persea americana* Mill.) is a globally consumed fruit. The cosmetic applications and documented anticancer, antibacterial, anti-inflammatory, and anti-hypertensive potentials of the peel

have been strongly linked to its antioxidant properties (Araujo *et al.*, 2021; Ferreira *et al.*, 2022). Phenolic acids, including hydroxybenzoic and hydroxycinnamic acids and flavonoids, including anthocyanins, flavanols, flavanones, and flavonols, are some of the classes of polyphenols reported in the avocado peel, which could contribute to its antioxidant effects (Tremocoldi *et al.*, 2018; Rodríguez-Martínez *et al.*, 2021; Lyu *et al.*, 2023). The radical scavenging activity of the peel extract has been documented (Tremocoldi *et al.*, 2018; Rodríguez-Martínez *et al.*, 2021; Rahman *et al.*, 2022; Lyu *et al.*, 2023). Also, the peel has been shown to exert anti-inflammatory effects in lipopolysaccharide (LPS)-treated murine macrophage cells by suppressing TNF- α expression and NO production (Tremocoldi *et al.*, 2018).

Despite the antioxidant and anti-inflammatory potential of avocado peel, previous studies have not been able to link or correlate its principal phytoconstituents to its bioactivity through comparative experimental analysis, which will give more insight into the antioxidant phytochemistry of the fruit's peel. In the present study, computational, *in vitro*, and cell-based experimental models were employed to elucidate the antioxidant phytochemistry of avocado fruit peel.

4.3 Materials and methods

Please refer to **sub-sections 3.1, 3.2, 3.3, 3.5 and 3.7 of chapter 3** for the materials and methods of this chapter.

4.4 Results

The methanol extract of the fruit peel had the highest ($p < 0.05$) total phenol, while the ethyl acetate extract had the highest ($p < 0.05$) total flavonoid content compared to the other extracts (Table 1). The Fe^{3+} reducing antioxidant activity of the methanol and dichloromethane extracts was significantly ($p < 0.05$) more potent than that of the other extracts and Trolox, but significantly ($p < 0.05$) less potent than that of ascorbic acid (Table 1). The DPPH, ABTS, and NO radical scavenging activity of the methanol extract were stronger than those of the other extracts and in some instances stronger and/or comparable to those of ascorbic acid and Trolox (**Table 4.1**).

Among the fractions (fractions 2 to 10) obtained from the methanol extract, fraction 8 consistently showed the highest ($p < 0.05$) total phenol content, as well as DPPH and ABTS radical scavenging and Fe^{3+} reducing antioxidant activity (Table 2). The antioxidant activity of fraction 8 was statistically comparable to that of ascorbic acid and/or Trolox (**Table 4.2**).

Table 4.1: Total phenol and flavonoid contents and *in vitro* Fe³⁺ reducing and radical scavenging activity of crude extract at 45 µg/mL.

Groups	Total Phenol Content (mg/g GAE)	Total Flavonoid Content (mg/g QE)	Fe ³⁺ Antioxidant Capacity (mg/g GAE)	DPPH Radical Scavenging Activity (%)	ABTS Radical Scavenging Activity (%)	NO Radical Scavenging Activity (%)
DCM	612 ± 48.2 ^b	6.94 ± 0.76 ^a	163 ± 0.61 ^b	68.1 ± 5.80 ^b	55.0 ± 5.31 ^b	52.9 ± 2.26 ^d
EtAc	142 ± 3.60 ^a	15.8 ± 2.24 ^b	31.8 ± 1.95 ^a	29.1 ± 1.26 ^a	31.8 ± 3.98 ^a	33.4 ± 4.78 ^c
MeOH	829 ± 48.6 ^c	8.17 ± 0.60 ^a	162 ± 0.42 ^b	93.9 ± 2.79 ^c	96.3 ± 3.14 ^c	62.6 ± 3.03 ^d
Water	157 ± 2.62 ^a	7.49 ± 0.19 ^a	38.7 ± 0.16 ^a	35.2 ± 1.80 ^a	40.8 ± 0.96 ^a	29.8 ± 6.51 ^{bc}
Asc	N/A	N/A	253 ± 28.1 ^c	87.5 ± 5.86 ^c	99.8 ± 0.16 ^c	11.1 ± 3.46 ^a
Trolox	N/A	N/A	57.1 ± 4.77 ^a	94.6 ± 1.25 ^c	98.1 ± 3.10 ^c	20.0 ± 2.21 ^{ab}

Abbreviations: “ABTS”, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); “Asc”, ascorbic acid; “DCM”, di-chloromethane extract; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl; “EtAc”, ethyl acetate extract; “GAE”, gallic acid equivalent; “MeOH”, methanol extract; “N/A”, not applicable; “NO”, nitric oxide; “QE”, quercetin equivalent. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Table 4.2: Total phenol and flavonoid contents and *in vitro* Fe³⁺ reducing and radical scavenging activity of the fractions from the methanol crude extract at 25 µg/mL.

Groups	Total Phenol Content (mg/g GAE)	Total Flavonoid Content (mg/g QE)	DPPH Radical Scavenging (%)	ABTS Radical Scavenging (%)	Fe ³⁺ Antioxidant Capacity (mg/g GAE)
F2	195 ± 1.42 ^{ab}	17.1 ± 4.00 ^{ab}	44.2 ± 1.57 ^{bc}	13.7 ± 0.21 ^b	24.8 ± 3.75 ^{ab}
F3	238 ± 12.5 ^{bc}	47.3 ± 4.10 ^{cd}	55.6 ± 1.03 ^c	40.9 ± 3.03 ^f	45.2 ± 2.52 ^b
F4	175 ± 4.21 ^a	84.45 ± 12.4 ^c	40.8 ± 1.37 ^{ab}	44.5 ± 0.03 ^f	46.4 ± 0.51 ^b
F5	236 ± 10.6 ^{bc}	8.04 ± 3.05 ^a	32.5 ± 5.6 ^a	30.1 ± 0.65 ^e	29.6 ± 0.51 ^{ab}
F6	252 ± 10.1 ^{cd}	29.3 ± 8.10 ^{bc}	43.2 ± 5.64 ^{ab}	22.5 ± 1.53 ^d	30.6 ± 6.49 ^{ab}
F7	339 ± 3.60 ^e	63.7 ± 14.9 ^{de}	68.8 ± 1.26 ^d	6.57 ± 0.01 ^a	25.6 ± 1.26 ^{ab}

F8	519 ± 48.6 ^f	39.4 ± 5.28 ^{bc}	90.8 ± 9.10 ^e	89.4 ± 4.65 ^g	96.7 ± 15.9 ^c
F9	288 ± 11.2 ^d	7.00 ± 0.40 ^a	51.9 ± 2.87 ^{bc}	15.4 ± 0.65 ^{bc}	19.0 ± 2.83 ^a
F10	179 ± 18.7 ^a	23.6 ± 7.47 ^{ab}	44.7 ± 0.33 ^b	20.6 ± 1.32 ^{cd}	44.3 ± 17.7 ^b
Asc	N/A	N/A	85.5 ± 1.32 ^e	99.4 ± 0.15 ^h	ND
Trolox	N/A	N/A	90.5 ± 2.52 ^e	99.9 ± 0.06 ^h	89.1 ± 13.0 ^c

Abbreviations: “ABTS”, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); “Asc”, ascorbic acid; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl; “F2” to “F10”, fraction 2 to fraction 10; “GAE”, gallic acid equivalent; “N/A”, not applicable; “ND”, not determined; “QE”, quercetin equivalent. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

LC-MS analysis of fraction 8 showed the presence of several phenolic compounds, with chlorogenic acid (5-CQA) and 1-*O*-caffeoylquinic acid (1-CQA) being the compounds with the highest concentrations (**Table 4.3** and **Figure 4.1**). Chlorogenic acid (5-CQA) was the compound of interest in this study.

Table 4.3: LC-MS data of fraction 8 from the methanol extract of avocado peel.

Identified Compounds	Ontology	ART (min)	AMz	Average Conc. (mg/L)
4-Hydroxy-3-prenylbenzoic acid glucoside	Phenolic glycosides	3.176	413.14206	0.1
Microperfurane	Butenolides	3.423	265.08621	0.5
Eudesmic acid	Gallic acid and derivatives	4.359	211.05984	0.1
Butalactin	Gamma butyrolactones	4.554	241.07141	0.1
Chlorogenic acid	Quinic acids and derivatives	4.72	353.08762	103.5
4-Hydroxybenzoic acid	Hydroxybenzoic acid derivatives	4.917	137.02409	0.1
Mycophenolic acid	Phthalides	5.014	319.12024	0.1
1- <i>O</i> -caffeoylquinic acid	Quinic acids and derivatives	5.029	353.0867	102.3
Caffeic acid	Hydroxycinnamic acids	5.164	179.0349	1.0
Quercetin 3-lathyroside	Flavonoid-3- <i>O</i> -glycosides	5.445	595.1261	21.9
Rutin	Flavonoid-3- <i>O</i> -glycosides	5.671	609.14667	1.0
Quercetin 3-galactoside	Flavonoid-3- <i>O</i> -glycosides	5.816	463.08737	30.0
Crepidiaside A	<i>O</i> -glycosyl compounds	5.868	421.14926	0.1
Gibberellin A17	C20-gibberellin 20-carboxylic acids	6.048	377.16031	0.1

Quercetin 3-[rhamnosyl-(1->2)-alpha-L-arabinopyranoside]	Flavonoid-3-O-glycosides	6.055	579.13745	14.2
Quercitrin	Flavonoid-3-O-glycosides	6.145	447.09045	43.4
Azelaic acid	Medium-chain fatty acids	6.41	187.09761	16.2
3-[4-[1-(4-Hydroxy-3-methoxyphenyl)-1,3-dihydroxyisopropoxy]-2-hydroxyphenyl]propyl alpha-L-rhamnopyranoside	Lignan glycosides	6.426	509.20142	0.1
Quercetin	Flavonols	6.978	301.03497	3.0
Corchorifatty acid F	Lineolic acids and derivatives	7.748	327.21799	0.1
Acrostalidic acid	Naphthopyrans	10.332	277.1442	5.4
Kopetdaghin A	Sesquiterpenoids	10.488	383.22238	0.5
Xanthoxin	Sesquiterpenoids	11.134	249.14981	16.5
etretinate	Retinoid esters	11.38	353.21255	4.2
3-Oxo-4,6-choladienoic acid	Bile acids, alcohols and derivatives	12.203	369.24011	3.4
1,1'-[1,11-Undecanediylbis(oxy)]bisbenzene	Phenol ethers	13.462	339.23151	108.8
Canrenone	Steroid lactones	14.821	339.19983	10.9

Abbreviations: “ART”, average retention time; “AMz”, average mass-to-charge ratio; “Conc.”, concentration.

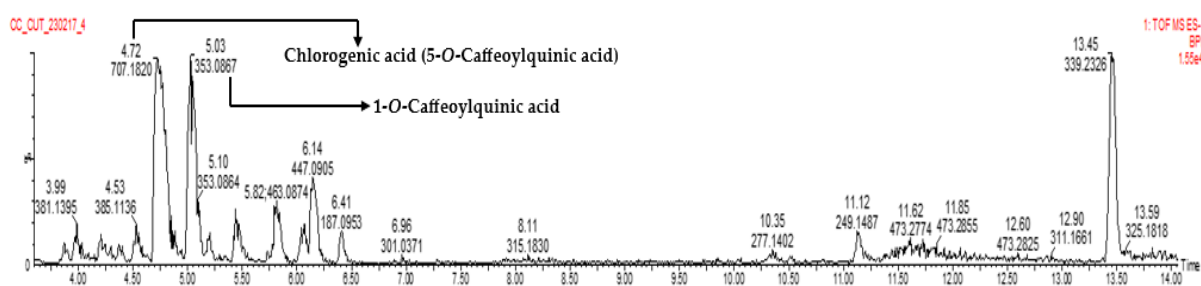


Figure 4.1: LC-MS chromatogram of fraction 8 from the methanol extract of avocado peel.

Dose-dependently, the DPPH radical scavenging activity of fraction 8 was statistically ($p > 0.05$) as potent as chlorogenic acid, ascorbic acid, and Trolox (**Table 4.4** and **Figure 4.2a**). Although not significantly ($p > 0.05$), the inhibitory effect of fraction 8 ($IC_{50} = 5.73 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 6.17 \mu\text{g/mL}$) on FS-induced linoleic acid peroxidation was stronger than that of ascorbic acid ($IC_{50} = 8.41 \mu\text{g/mL}$) (**Table 4.4** and **Figure 4.2b**).

Table 4.4: IC₅₀ values for the dose-dependent activities of the tested samples.

Assays	Fraction 8	Ascorbic Acid	Trolox	Chlorogenic Acid
	IC ₅₀ Values (µg/mL)			
DPPH radical scavenging	5.59 ± 1.75 ^{ab}	6.50 ± 2.18 ^{ab}	2.41 ± 0.46 ^b	8.84 ± 1.44 ^a
Anti-linoleic acid peroxidation	5.73 ± 1.10	8.41 ± 3.33	ND	6.17 ± 1.26
Anti-lipid peroxidation in Chang cells	15.9 ± 3.16 ^a	5.74 ± 0.66 ^b	ND	9.34 ± 2.40 ^b
Inhibition of ROS production in RAW 264.7 cells	39.6 ± 3.05 ^a	8.08 ± 1.83 ^c	ND	28.2 ± 2.50 ^b
Inhibition of NO production in RAW 264.7 cells	63.5 ± 4.89 ^c	203 ± 26.8 ^a	ND	107 ± 2.06 ^b

“ND” means “not determined”. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters attached to each data represent significant differences ($p < 0.05$) between groups when there are no similar letters.

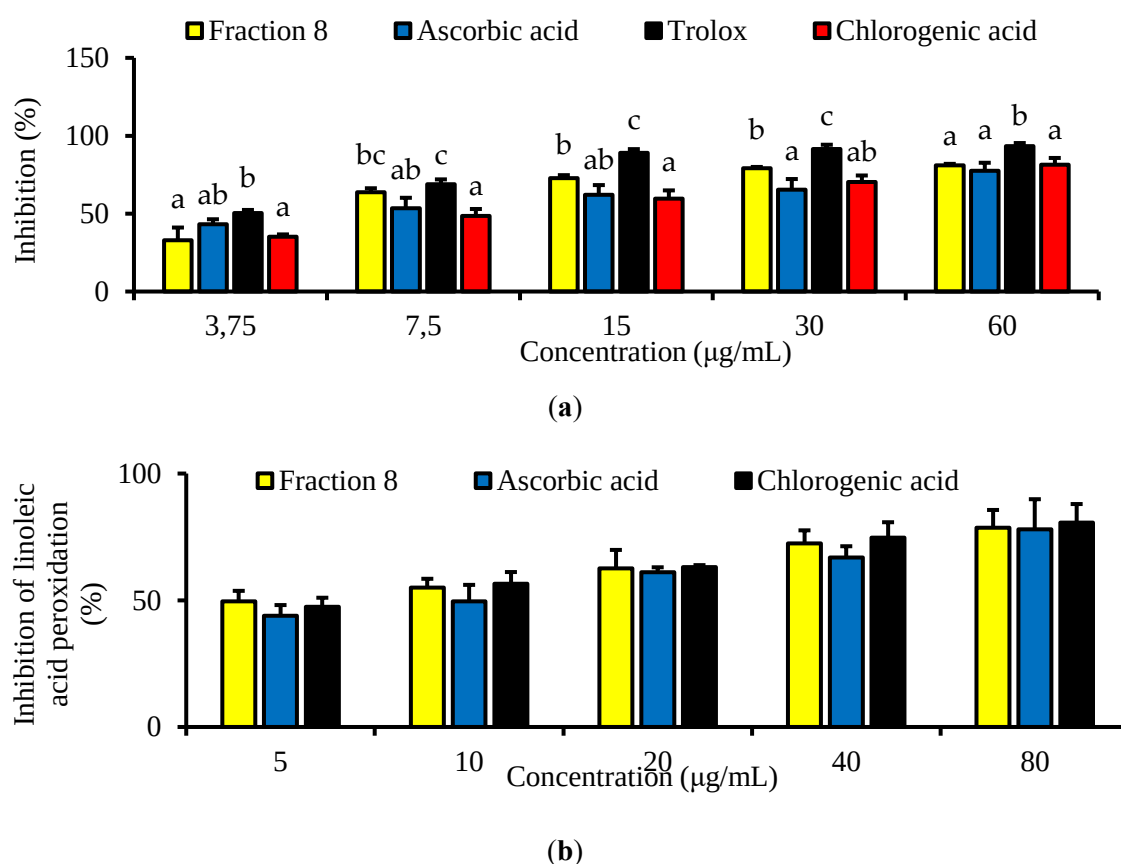


Figure 4.2: Dose-dependent (a) DPPH radical scavenging and (b) anti-linoleic acid peroxidative activities of fraction 8 and chlorogenic acid. Data are presented as mean ± SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters.

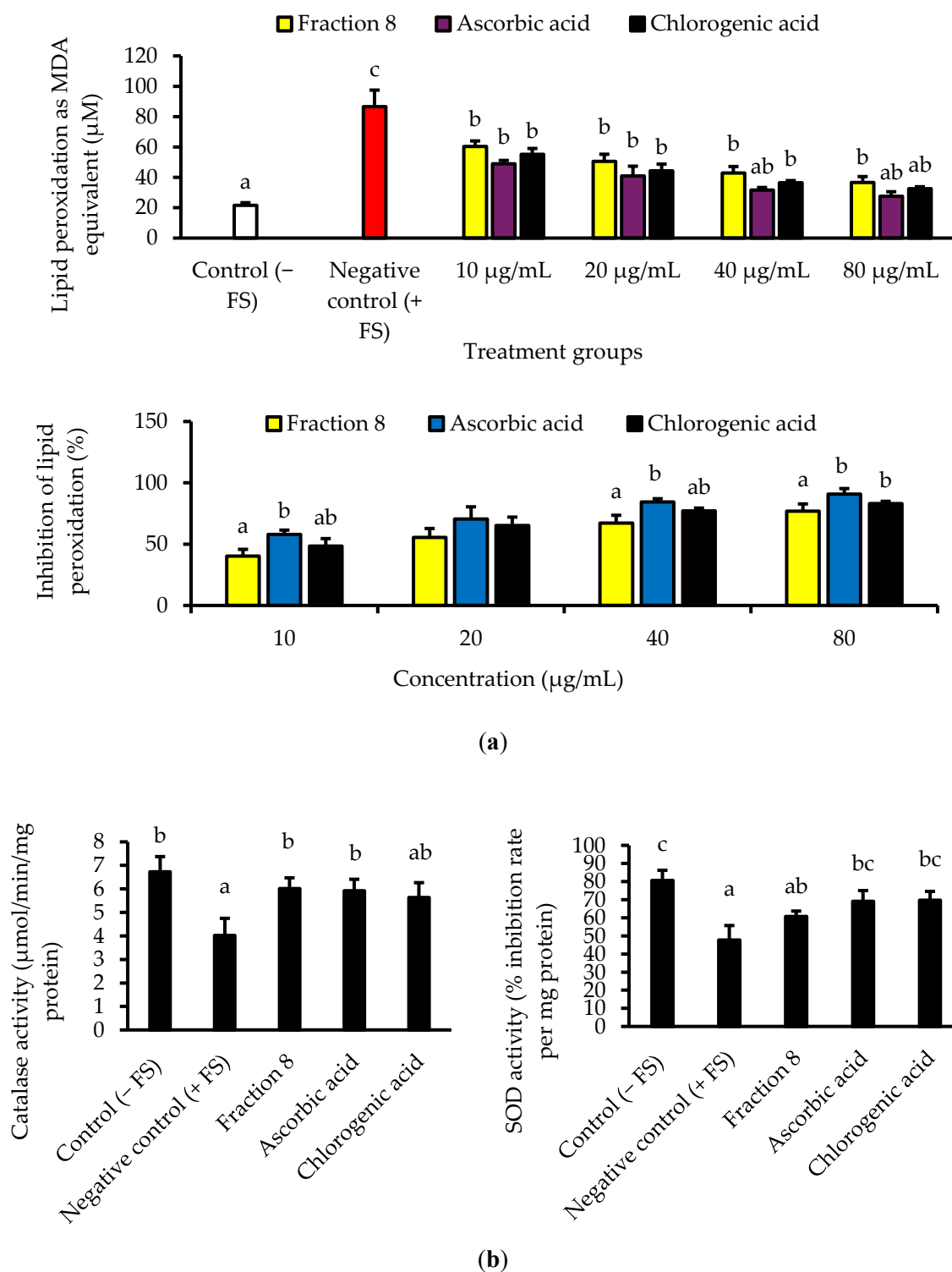


Figure 4.3: (a) Dose-dependent anti-lipid peroxidative and (b) antioxidant enzyme modulatory activities of fraction 8 and chlorogenic acid in FS-treated Chang cells. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done between the controls (control and negative control) and treatment groups at a given concentration or among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters. “FS” means $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

In Chang cells induced with oxidative stress, the anti-lipid peroxidative of ascorbic acid ($IC_{50} = 5.74 \mu\text{g/mL}$) was significantly ($p < 0.05$) and non-significantly ($p > 0.05$) stronger than that of fraction 8 ($IC_{50} = 15.9 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 9.34 \mu\text{g/mL}$), respectively (**Table 4.4** and **Figure 4.3a**). Nevertheless, fraction 8, chlorogenic acid, and ascorbic acid (at $80 \mu\text{g/mL}$) appreciably mitigated oxidative stress-induced depletion of catalase and SOD enzymes in the Chang cells (**Figure 4.3b**).

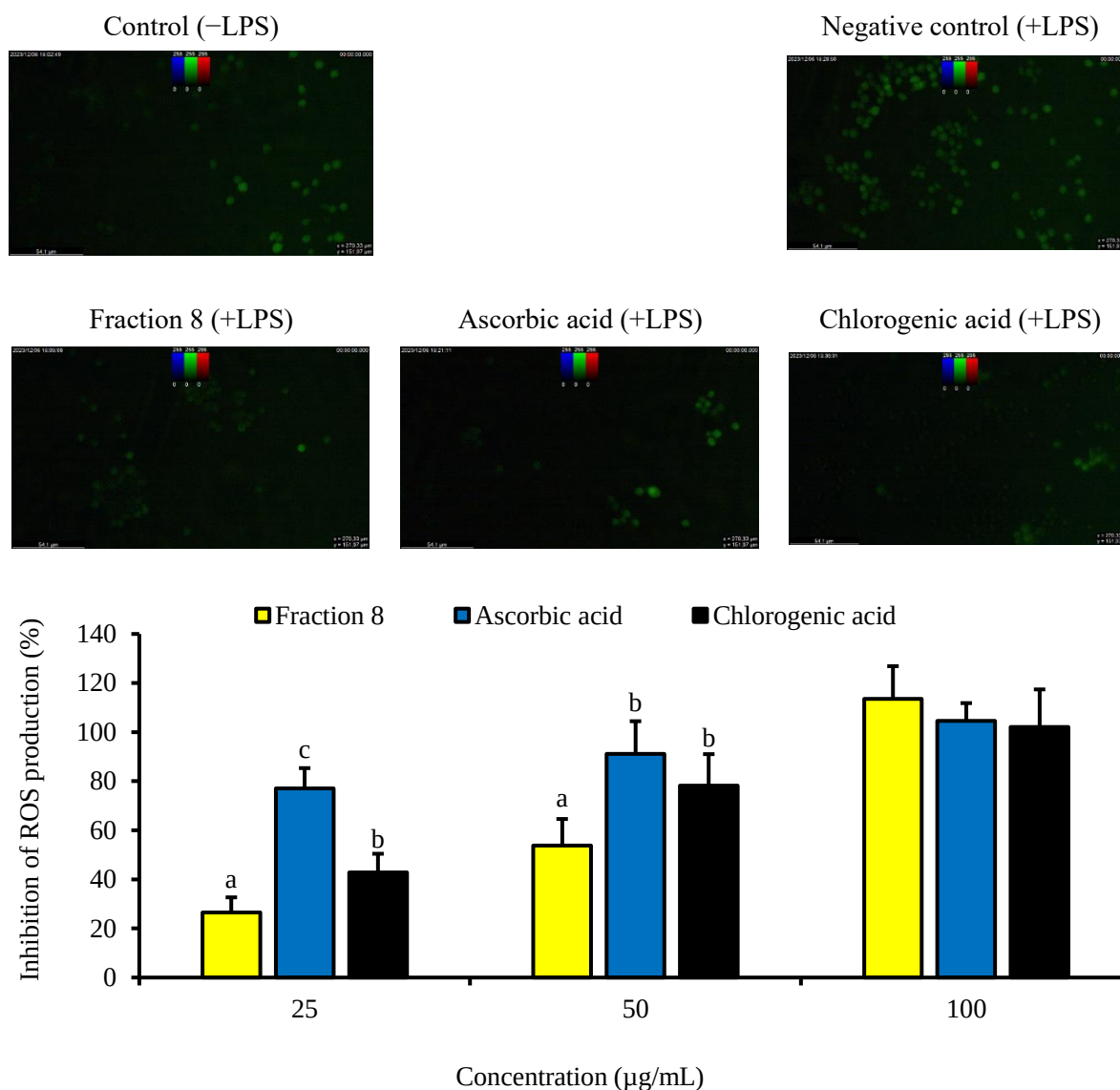


Figure 4.4: Dose-dependent inhibitory effect of fraction 8 and chlorogenic acid on ROS production in LPS-treated RAW 264.7 macrophages. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters. “LPS” means lipopolysaccharide.

Although not as potent as ascorbic acid ($IC_{50} = 8.08 \mu\text{g/mL}$; $p < 0.05$), fraction 8 ($IC_{50} = 39.6 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 28.2 \mu\text{g/mL}$) showed promising inhibition of LPS-induced ROS production in RAW 264.7 macrophages (**Table 4.4** and **Figure 4.4**). However, the ability of fraction 8 ($IC_{50} = 63.5 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 107 \mu\text{g/mL}$) to inhibit NO production in LPS-treated RAW 264.7

macrophages significantly ($p < 0.05$) outperformed that of ascorbic acid ($IC_{50} = 203 \mu\text{g/mL}$) (Table 4.4 and Figure 4.5a). Interestingly, both fraction 8 and chlorogenic acid were not toxic to the RAW 264.7 macrophages (Figure 4.5b).

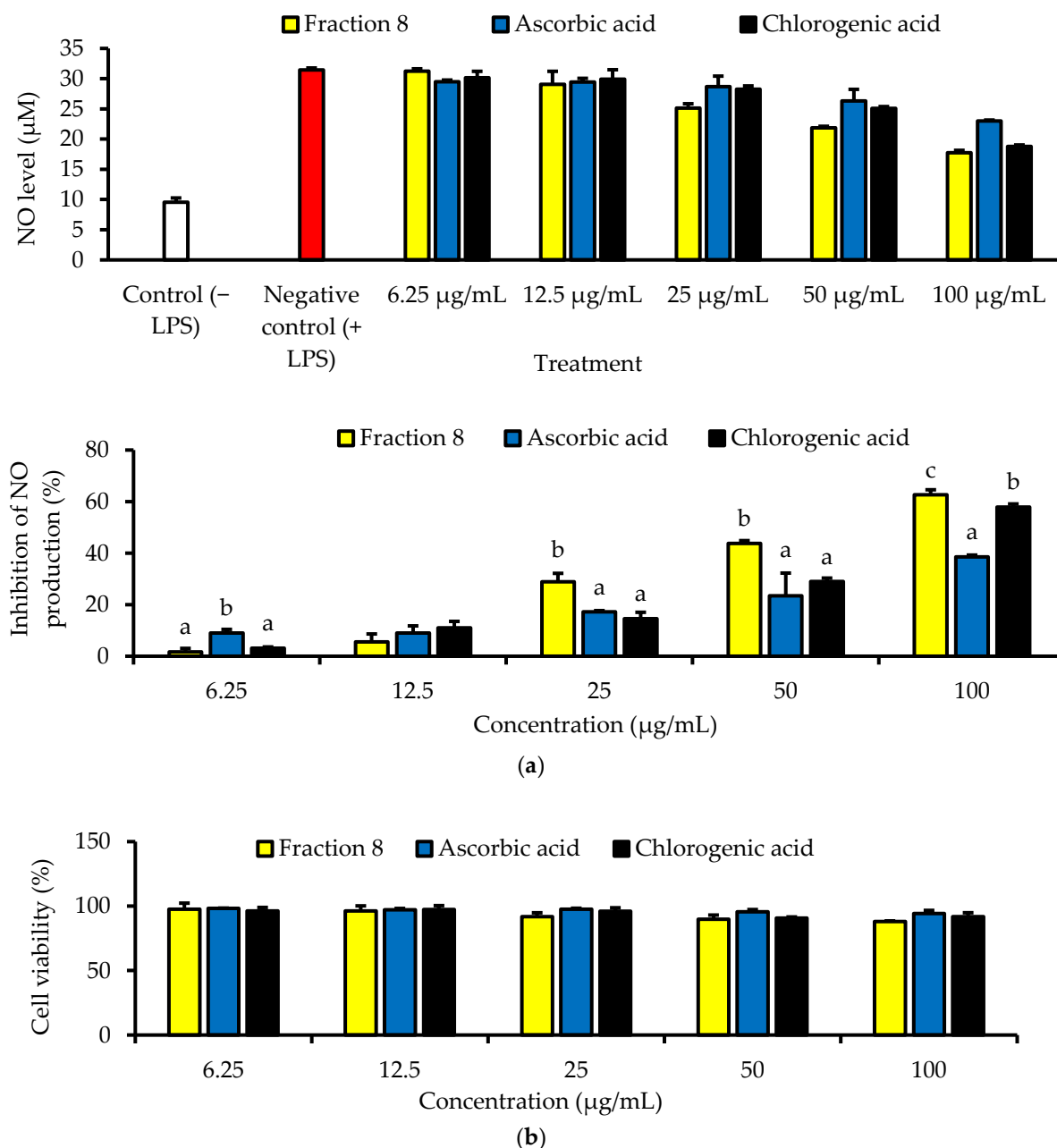


Figure 4.5: Dose-dependent inhibitory effect of fraction 8 and chlorogenic acid on (a) NO production in LPS-treated RAW 264.7 macrophages and (b) the viability of RAW 264.7 macrophages. Data are presented as mean $\pm$ SD of triplicate analysis. Data are presented as mean $\pm$ SD of triplicate analysis. Statistical comparison was done between the controls (control and negative control) and treatment groups at a given concentration or among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters. “LPS” denotes “lipopolysaccharide”.

The Density Functional Theory (DFT) analysis data are presented in **Table 4.5** and **Figure 4.6**. **Figure 4.6a** depicts the chemical structure of 5-CQA and the selected hydroxyl group numbering, as well as the lowest energy structural conformer of chlorogenic acid (5-CQA). **Figure 4.6b** shows the electron spin density of the optimized structures of 5-CQA radicals for HAT mechanism of DPPH radical scavenging.

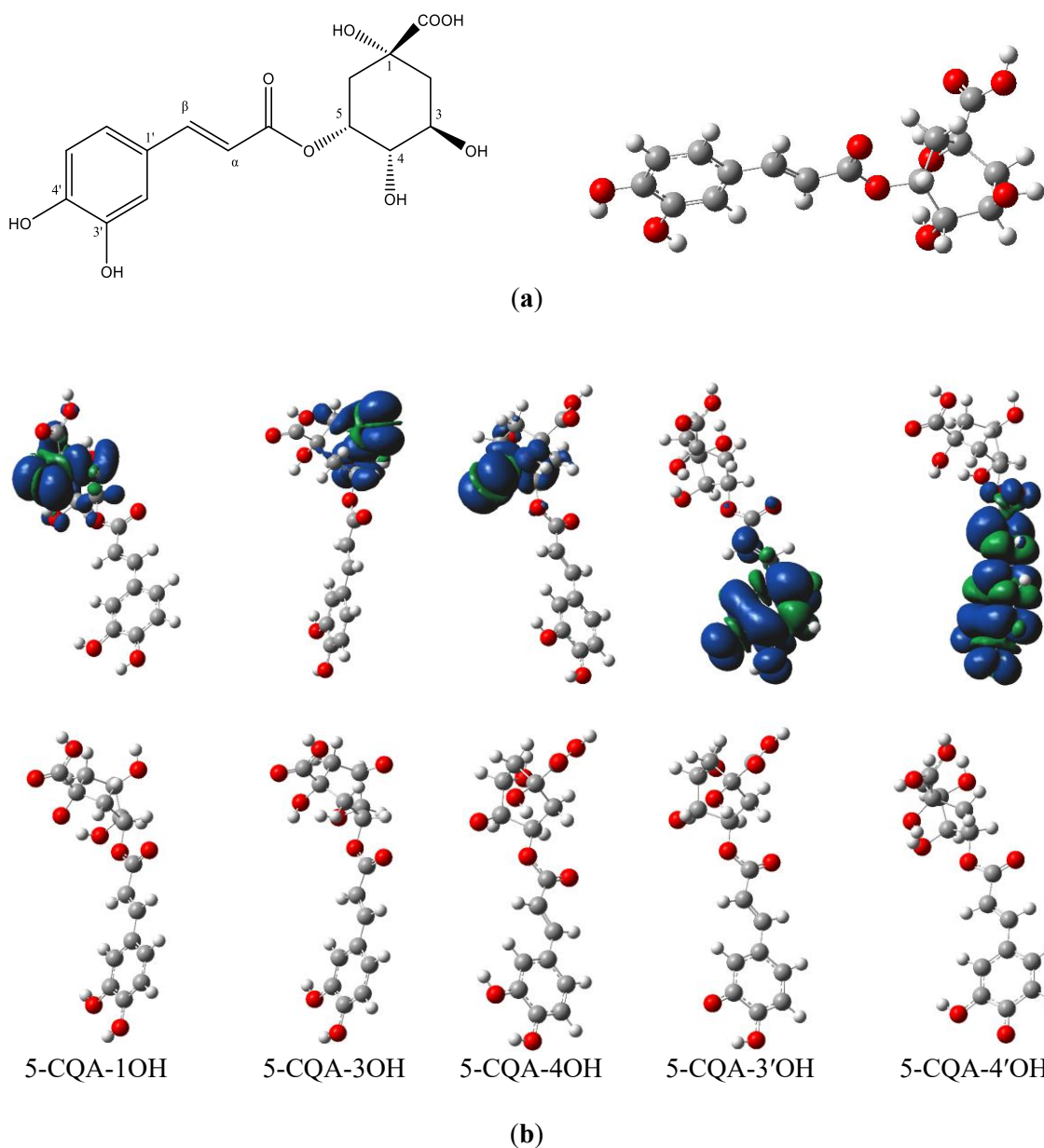


Figure 4.6: (a) Chemical structure of chlorogenic acid (5-*O*-caffeoylquinic acid) and its lowest-energy structural conformer; (b) the electron spin density of the optimized structures of 5-*O*-caffeoylquinic acid radicals.

For the HAT mechanism, 5-CQA-4'OH H-atom abstraction appears to be the favoured mechanism for quenching DPPH radical according to the low BDE value (**Table 4.5**) and stable electron distribution on the phenolic radical (**Figure 4.6b**). For the SET-PT mechanism, the proton transfer step is more

highly favoured at positions 3' and 4' based on the low PDE values (**Table 4.5**). Overall, the SET-PT mechanism at the 5-CQA-3'OH and 5-CQA-4'OH positions appear to be the preferred mechanism for DPPH radical scavenging compared to the HAT and SPLET mechanisms due to the low enthalpy values (**Table 4.5**)

Table 4.5: Bond dissociation enthalpy (*BDE*), adiabatic ionization potential (*AIP*), proton dissociation enthalpy (*PDE*), proton affinity (*PA*), and electron transfer enthalpy (*ETE*) obtained (in kcal/mol) of the reaction species for the capture of the DPPH radical via 5-CQA.

Structure *	HAT	SET-PT		SPLET	
	BDE	AIP	PDE	PA	ETE
	kcal/mol				
5-CQA-1OH	27.6	22.0	5.6	14.0	13.6
5-CQA-3OH	24.7	22.0	2.6	22.1	2.6
5-CQA-4OH	27.1	22.0	5.0	24.1	3.0
5-CQA-3'OH	2.6	22.0	-19.4	5.0	-2.4
5-CQA-4'OH	2.1	22.0	-19.9	5.3	-3.2

* The numbers 1, 3, 4, 3', and 4' denote the position of the eliminated hydrogen from 5-CQA.

4.5 Discussion

Oxidative stress is pivotal in the pathological outcomes of many diseases, which has been linked to the oxidative damage caused by deleterious ROS on biological molecules (Jomova *et al.*, 2023). The peel of avocado fruit has been shown to have antioxidant effects (Tremocoldi *et al.*, 2018; Rodríguez-Martínez *et al.*, 2021; Lyu *et al.*, 2023). In this study, we presented the antioxidant phytochemistry of avocado fruit peel.

The highly reactive nature of biological radicals notably influences their ability to oxidatively damage biomolecules. Plant polyphenols, on the other hand, are known radical scavengers, due to their ability to transfer hydroxyl H-atom and form stable phenoxy radicals (Chen *et al.*, 2020). Also, the iron-chelating ability of polyphenols contributes to their antioxidant potential (Scarano *et al.*, 2023). In the present study, the methanol extract of avocado fruit peel had the highest polyphenol content and consistently had the highest radical scavenging and Fe³⁺ reducing activity relative to the other extracts (**Table 4.1**). Although not on the same variety, previous studies have documented the potent radical scavenging effects of the peel's methanol extract (Antasionasti *et al.*, 2017; Rahman *et al.*, 2022). Consistent with our study, the methanol extract was more potent than ethyl acetate extract (Antasionasti *et al.*, 2017). Among all the fractions from the methanol extract, fraction 8 had the most potent radical scavenging and Fe³⁺ reducing activity (**Table 4.2**). The activity correlates with the total phenol content

(**Table 4.2**), confirming the influence of polyphenols on the radical quenching potential of plants. Supporting documented evidence has also shown correlations between the phenolic contents of avocado peel extract and its radical scavenging and in vitro antioxidant capacity (Antasionasti *et al.*, 2017; Lyu *et al.*, 2023). In fact, relative to the pulp and seed, studies have shown that the peel possessed more phenolic content, and thus had stronger radical scavenging and antioxidant capacity (Hirasawa *et al.*, 2008; Rojas-García *et al.*, 2022; Lyu *et al.*, 2023).

Previous studies have reported the presence of phenolic acids, including hydroxybenzoic and hydroxycinnamic acids and flavonoids, including anthocyanins, flavanols, flavanones, and flavonols in avocado peel (Tremocoldi *et al.*, 2018; Rodríguez-Martínez *et al.*, 2021; Lyu *et al.*, 2023), which were partly identified in fraction 8 (**Table 4.3**). Also, the phenol content of fraction 8 was higher than the flavonoid content (**Table 4.2**), which suggests phenolic compounds may have influenced its antioxidant capacity more than the flavonoids. A consistent trend of data has previously been reported in fractions obtained from the methanol and ethyl acetate extracts of peel of an avocado variety grown in Ampelgading, Indonesia (Scarano *et al.*, 2023). It was shown that the phenolic content contributed more to the radical scavenging and ferric-reducing activity of the fractions than the flavonoids content (Scarano *et al.*, 2023). In the study, 1,2,4-trihidroksiheptadek-12,16-diyne and 1,2,4-trihidroksiheptadek-16-yne-18-ene were identified as the possible antioxidant compounds in the fraction obtained from the methanol and ethyl acetate extracts, respectively (Scarano *et al.*, 2023). However, this study (Scarano *et al.*, 2023), like other studies (Tremocoldi *et al.*, 2018; Rodríguez-Martínez *et al.*, 2021, Lyu *et al.*, 2023), did not compare the antioxidant activity of the identified compounds with that of the potent extracts or fractions in which they were identified. Thus, the study was not able to ascertain whether the identified compounds influence the antioxidant capacity of the peel extracts or fractions. This suggests a gap in these studies and provides the rationale and relevance of our study.

In our study, there was a predominant presence of chlorogenic and 1-*O*-caffeoylquinic acid in fraction 8 (**Table 4.3**). Chlorogenic acid and derivatives have been reported in avocado peel (Hirasawa *et al.*, 2008; Rodríguez-Martínez *et al.*, 2021). The data of our study further showed that the radical scavenging activity chlorogenic acid was comparable to that of fraction 8 and ascorbic acid (**Table 4.4** and **Figure 4.2a**), suggesting that chlorogenic acid may strongly influence the antioxidant effect of fraction 8. Moreover, chlorogenic acid is a known dietary phenolic acid with antioxidant and anti-inflammatory potentials (Liang *et al.*, 2015; Wang *et al.*, 2022; Huang *et al.*, 2023). Further computational DFT analysis suggests that the favoured radical scavenging mechanism of chlorogenic acid is a single electron transfer followed by proton transfer at 3'OH and 4'OH positions (**Table 4.5** and **Figure 4.6**).

Furthermore, the radical scavenging potency of fraction 8 and chlorogenic acid (**Table 4.4**) may be relevant in their anti-lipid peroxidative attributes. This is because during lipid peroxidation free radicals can oxidatively attack biological lipids, including membrane lipids, compromising cell integrity, and exacerbating oxidative stress (Petrovic *et al.*, 2020). In vitro and in Chang cells, fraction 8 and chlorogenic acid dose-dependently inhibited lipid peroxidation (**Table 4.4** and **Figures 4.2b** and **4.3a**). In Chang cells, the anti-lipid peroxidative effect of fraction 8 and chlorogenic acid was accompanied by the modulation of SOD and catalase activities (**Figure 4.3b**), which are key antioxidant enzymes. The anti-lipid peroxidative and antioxidant enzyme modulatory effect of chlorogenic acid was comparable to that of ascorbic acid and fraction 8, suggesting chlorogenic acid may notably influence the anti-lipid peroxidative effect of fraction 8. Moreover, the anti-lipid peroxidative and antioxidant enzyme modulatory effect of chlorogenic acid has been documented in animal disease models (Zhou *et al.*, 1993; Wang *et al.*, 2020).

In LPS-treated RAW 264.7 macrophages, fraction 8 and chlorogenic acid dose-dependently suppressed oxidative stress by suppressing ROS production (**Table 4.4** and **Figure 4.4**). This data, together with the potent anti-lipid peroxidative and antioxidant enzyme modulatory action of chlorogenic acid (**Figure 4.3**) strongly supports the documented modulatory action of chlorogenic acid on biological redox status (Zhou *et al.*, 1993; Liang *et al.*, 2015; Wang *et al.*, 2020; Wang *et al.*, 2022) and further supports its notable contribution to the antioxidant properties of avocado peel. Furthermore, both fraction 8 and chlorogenic acid dose-dependently suppressed NO production in LPS-treated RAW 264.7 macrophages (**Table 4.4** and **Figure 4.5a**) without causing cytotoxicity (**Figure 4.5b**). In fact, they outperformed ascorbic acid. Immune myeloid cells, like macrophages, are known to produce more cytokines and NO in response to inflammatory signals (Palmieri *et al.*, 2020). Thus, the inhibitory action of fraction 8 on NO production in RAW 264.7 macrophages induced with inflammation suggests an anti-inflammatory potential. Through the suppressive action on LPS-induced NO production and TNF- α expression in RAW 264.7 macrophages, existing data have shown the anti-inflammatory action of the hydroalcoholic extract of avocado peel (Tremocoldi *et al.*, 2018). Data from our study further shows that the anti-inflammatory potential of avocado peel may be strongly influenced by the presence of chlorogenic acid, considering the promising suppressive effect of chlorogenic acid on LPS-induced NO production (**Table 4.4** and **Figure 4.5a**). Moreover, chlorogenic acid has been reported to potentiate anti-inflammatory action by moderating the production and action of inflammatory mediators, including TNF- α , IL-1 β , IL-6, IL-8, NO, and PGE2, while modulating signalling processes associated with NF- κ B, MAPK, Nrf2, etc. (Huang *et al.*, 2023).

4.6 Conclusion

In this study, a bioassay-guided approach was used to elucidate the antioxidant phytochemistry of avocado peel. Fractionation of the peel's methanol extract yielded a fraction that had a promising ability to scavenge free radical, inhibit lipid peroxidation, suppress ROS and NO production and modulate antioxidant enzyme activity. The fraction also showed the predominant presence of chlorogenic acid. Data further showed that chlorogenic acid had antioxidant activities that were comparable to that of the fraction and ascorbic acid. It scavenged DPPH radical through a single electron transfer, followed by proton transfer at its carbons 3'OH and 4'OH positions. Considering that our investigation was bioassay-guided, it is logical to conclude that chlorogenic acid is a bioactive principle that notably influences the antioxidant capacity of avocado fruit peel.

CHAPTER 5

EPICATECHIN ISOLATED FROM *LITCHI CHINENSIS* SONN. (LITCHI) FRUIT PEEL ETHYL ACETATE EXTRACT MODULATED GLUCOSE UPTAKE IN CHANG CELLS AND SUPPRESSED ROS PRODUCTION IN RAW 264.7 MACROPHAGES

Prescript

This chapter investigated and reported the antioxidant and glycaemic control assay-guided isolation of bioactive epicatechin from the ethyl acetate extract of litchi fruit peel. The chapter has been published in **Antioxidants** as follows: ***Izu GO, Mapasa NP, Namboozee J, Chukwuma MS, Njoya EM, Tabakam GT, Bonnet SL, Makhafola TJ, Mashele SS, Chukwuma CI*. (2024). Epicatechin Isolated from Litchi chinensis Sonn. (Litchi) Fruit Peel Ethyl Acetate Extract Modulated Glucose Uptake in Chang Cells and Suppressed ROS Production in RAW 264.7 Macrophages. Antioxidants, 13(10): 1233. <https://doi.org/10.3390/antiox13101233>***. The above-mentioned publication was adopted in writing the content of this chapter.

5.1 Abstract

Bioactive flavonoid, epicatechin, has been reported in the peel of litchi fruit, but isolated from its hydroalcoholic extracts. This study isolated epicatechin with cellular glucose uptake modulatory and ROS production inhibitory properties from the ethyl acetate (EtOAc) extract using a bioassay-guided approach. The fruit peel was defatted with hexane and sequentially extracted using dichloromethane (DCM), EtOAc, methanol (MeOH), and water. *In vitro*, phytochemical models, namely antioxidant (Fe^{3+} reducing, radical scavenging, and anti-linoleic acid peroxidative) and glycaemic control (α -glucosidase and α -amylase inhibitory and glucose uptake modulatory), were employed for the bioassay-guided isolation, while the isolated compound was characterized using NMR and mass spectrometry and assessed for dose-dependent inhibition of α -glucosidase and lipopolysaccharide (LPS)-induced cellular ROS production, as well as modulation of cellular glucose uptake. Relative to the other extracts, the EtOAc extract had appreciable phenol and flavonoid contents, which perhaps influenced its potent anti-lipid peroxidative (65.0%) and α -glucosidase inhibitory (52.4%) effects. The α -glucosidase inhibitory potency of the fractions (1 – 8) from the EtOAc extract correlated with their flavonoid contents, with fraction 5 outperforming other fractions. The fraction comprised a pool of fractions obtained from the DCM:MeOH:water (7:3:0.281 v/v/v) solvent system. LC-MS revealed the predominant presence of epicatechin in fraction 5, which was later isolated from one of the sub-fractions (sub-fraction 4) of fraction 5. This sub-fraction had stronger anti-lipid peroxidative (65.5%), α -glucosidase inhibitory (65.8%) and glucose uptake modulatory (38.2%) effects than the other sub-fractions from fraction 5, which could have been influenced by the isolated epicatechin. Moreover, the isolated epicatechin inhibited α -glucosidase ($\text{IC}_{50} = 35.3 \mu\text{M}$), modulated cellular glucose uptake ($\text{EC}_{50} = 78.5 \mu\text{M}$) and inhibited LPS-induced ROS production in RAW 264.7 macrophages in a dose-dependent fashion [$\text{IC}_{50} = 18.9 \mu\text{M}$; statistically comparable ($p > 0.05$) to ascorbic acid, $\text{IC}_{50} = 9.57 \mu\text{M}$]. Epicatechin from litchi peel EtOAc extract could potentiate glucose uptake modulatory, α -glucosidase inhibitory and ROS suppressive capacities, which could be influential in the use of litchi fruit peel for managing diabetes and associated oxidative damage.

Key words: *Antioxidant; epicatechin; glucose uptake; isolation; litchi peel.*

5.2 Introduction

Diabetes remains a global health concern as it detrimentally affects the socio-economic sphere of many countries around the world. This has been aggravated by its ever-increasing prevalence, projected to increase from 537 million adults in 2021 to 783 million adults in 2045 (IDF, 2021). The disease is classically characterized by high blood glucose caused by abnormal nutrient metabolism. Type 2 diabetes (T2D) remains the most prevalent type of diabetes (IDF, 2021), possibly because it is influenced by lifestyle-related factors, including dieting, weight gain, and physical inactivity (Zhu *et*

al., 2021). Insulin resistance is the main pathologic condition in T2D. Typically, it causes impaired metabolism of glucose. This includes drastically reduced uptake of circulating glucose into active cells of peripheral tissues due to the non-responsiveness of these cells to insulin signalling, which contributes to hyperglycaemia (Galicia-Garcia *et al.*, 2020).

Hyperglycaemia-induced oxidative stress is pivotal in developing diabetic complications due to oxidative damage caused by reactive oxygen species (ROS) produced during persistent hyperglycaemia (Bhatti *et al.*, 2022). Notably, there is an elevated generation of superoxide radicals in respiring cells during hyperglycaemia (Bhatti *et al.*, 2022). Elevated radical production can trigger lipid peroxidation. ROS can oxidatively damage the lipid bilayer of cells through lipid peroxidation, comprising the integrity of the cells (Bhatti *et al.*, 2022). Moreover, lipid peroxidation processes can lead to the generation of more deleterious radical by-products, which can worsen oxidative stress. Furthermore, persistent hyperglycaemia can increase the rate of glycation of other biomolecules. This leads to the generation of advanced glycation end products (AGEs), biomarkers implicated in degenerative diseases like diabetes (Bhatti *et al.*, 2022).

While there are many medications for managing diabetes and associated oxidative complications, dietary phytochemicals are increasingly becoming popular supplements to mitigate oxidative stress and manage metabolic diseases (Sun *et al.*, 2020). Many fruits are believed to contain antioxidant phenolics that influence their health benefits (Afam *et al.*, 2021). *Litchi chinensis* Sonn. (Sapindaceae) is a tropical and subtropical fruit with high commercial value due to its global consumption. It is known to have nutritional value and medicinal benefits (Zhao *et al.*, 2020). Studies have shown that the fruit has antioxidant, anti-hyperglycaemic, anti-hypertensive, anti-obesogenic, anti-dyslipidaemic, and hepatoprotective potentials (Zhao *et al.*, 2020). Due to its commercial value, enormous amounts of by-products, including the peel and seeds, are generated from its processing, which contains bioactive phenolic compounds in their free and bound forms (Cano-Gómez *et al.*, 2024). Thus, the fruit's wastes could be of medicinal relevance.

In the context of antidiabetic potential, a previous review (Chukwuma *et al.*, 2021) has shown that more studies have been conducted on the seed of litchi fruit than the peel, warranting the need for more studies to probe the anti-diabetic potential of the peel. The few anti-diabetic studies that have been conducted suggest that fruit peel has glycaemic control potential. A study has shown that hydroalcoholic extracts of litchi peel have mild and strong inhibitory activity on α -amylase and α -glucosidase, respectively (Tanaka *et al.*, 2024), suggesting its potential to suppress postprandial glycaemia. In another study, the peel flour and procyanidin-rich fraction from the peel reduced blood glucose and improved blood lipid profile in high-fat-diet-fed rats and mice (Rong *et al.*, 2017; Queiroz *et al.*, 2018). A recent study further showed that litchi peel ethanol extract ameliorated T2D pathologies in *db/db* mice by reducing blood glucose, hepatic gluconeogenesis, oxidative stress, and associated inflammatory responses (Yang *et al.*,

2024). Phytochemical assessments suggest that several key flavonoids, including catechins, procyanidins, proanthocyanidins, quercetin, rutin, and their derivatives may be influential in the antioxidant and other pharmacological properties of litchi peel (Zhao *et al.*, 2006; Zhao *et al.*, 2007; Li *et al.*, 2007).

The trend of previous diabetes-related pharmacological studies on litchi peel suggests that the focus has mostly been on the hydroalcoholic extracts, with limited studies on other extracts, such as the ethyl acetate extract (Chukwuma *et al.*, 2021). Solvent extraction using ethyl acetate allows hydrogen bond formation with carboxyl and hydroxyl groups. It thus can result in the extraction of medium-polar to polar phenolics and/or flavonoids (Prasad *et al.*, 2010), which could influence potent medicinal properties. In fact, documented qualitative evidence has shown that litchi peel hydroalcoholic and ethyl acetate extracts have relatively similar proportions of phenolic compounds and flavonoids (Chauhan *et al.*, 2021). Concomitant pharmacological evidence in diabetic rats further suggests that the blood glucose lowering, and antioxidant effect of litchi peel ethyl acetate extract outperforms that of the hydroalcoholic extract (Chauhan *et al.*, 2021), suggesting that the ethyl acetate extract of litchi peel could also be a source of flavonoids with antioxidant and glycaemic control potential. However, this has not been explored and thus remains elusive. Previous studies reporting the isolation of some bioactive flavonoids (proanthocyanidin B4, proanthocyanidin B2, and epicatechin) from litchi peel were from the hydroalcoholic extract and not the ethyl acetate extract (Zhao *et al.*, 2006; Zhao *et al.*, 2007). Thus, there is a need to explore litchi peel ethyl acetate extract as a source of key bioactive flavonoids. Therefore, in this study, we, for the first time, isolated a key bioactive flavonoid (epicatechin) from litchi peel ethyl acetate extract using an antioxidant and glycaemic control assay-guided approach, thus providing an alternative optimum approach to recovering bioactive epicatechin from litchi peel and more insight into the antidiabetic and antioxidant phytochemistry of litchi peel.

5.3 Materials and methods

Please refer to **sub-sections 3.1, 3.2, 3.4, 3.5 and 3.7 of chapter 3** for the materials and methods of this chapter.

5.4 Results and discussion

The antidiabetic and antioxidant potential of litchi peel has been documented (Rong *et al.*, 2017, Queiroz *et al.*, 2018; Chukwuma *et al.*, 2021; Yang *et al.*, 2024; Tanaka *et al.*, 2024). However, these studies and the isolation of bioactive flavonoids (epicatechin and proanthocyanidins) from litchi peel, have mostly been done on the hydroalcoholic extracts (Zhao *et al.*, 2006; Zhao *et al.*, 2007; Rong *et al.*, 2017; Queiroz *et al.*, 2018; Chukwuma *et al.*, 2021; Tanaka *et al.*, 2024; Yang *et al.*, 2024). Documented

evidence suggests that the ethyl acetate extract of litchi peel may possess stronger *in vivo* antidiabetic and antioxidant potential than the hydroalcoholic extract (Chauhan *et al.*, 2021) and may be a source of the key bioactive flavonoids in litchi peel. However, the ethyl acetate extract of litchi peel has not been explored in this context. In this study, we used bioassay-guided isolation to demonstrate for the first time that the ethyl acetate extract of litchi peel can be an alternative source to recover epicatechin with cellular glucose uptake modulatory and ROS production suppressive capacities.

5.4.1 Phytochemical and bioactive profiles of the peel extracts

Extraction of the peel with DCM, EtOAc, MeOH and water yielded 17.3 (5.74%), 2.51 (0.84%), 63.1 (21.0%) and 12.6 g (4.20%) of extracts, respectively. The EtOAc extract had the highest ($p < 0.05$) total phenol content. In contrast, the MeOH and EtOAc extracts had significantly ($p < 0.05$) higher total flavonoid contents than the DCM and water extracts (**Table 5.1**). The rich phenolic and flavonoid profile of the EtOAc extract could be linked to its ability to form hydrogen bonds with carboxyl and hydroxyl groups. It thus can extract medium polar to polar phenolics and/or flavonoids (Prasad *et al.*, 2010). Perhaps, the phenolic and flavonoid profile of the EtOAc extract also influenced its FRAP, radical scavenging and anti-linoleic acid peroxidative activities, which were stronger than those of the other extracts and statistically comparable ($p > 0.05$) to those of ascorbic acid and/or Trolox (**Table 5.1**). Phenolics have the ability to form stable phenoxy radicals, which influences their ability to scavenge radicals and chelate metals, making them good antioxidants (Chen *et al.*, 2020, Scarano *et al.*, 2023). Moreover, qualitative phytochemical analysis of litchi peel EtOAc extract, and hydroalcoholic extracts has shown the presence of phenolic compounds and flavonoids in a relatively similar fashion and could be influential in the potent radical scavenging, FRAP and anti-lipid peroxidative activities demonstrated by the ethyl acetate and methanol extracts (**Table 5.1**) (Zhao *et al.*, 2006; Zhao *et al.*, 2007; Li *et al.*, 2007).

Table 5.1: Total phenol and flavonoid contents and *in vitro* Fe³⁺ reducing, radical scavenging, anti-linoleic acid peroxidative and enzyme inhibitory activities of crude extracts at 55 µg/mL.

Extracts and standards	TPC (mg/g GAE)	TFC (mg/g QE)	FRAP (mg/g GAE)	DPPH (%)	ABTS (%)	NO (%)	AnLP (%)	AGI (%)	AAI (%)
DCM	39.7 ± 1.35 ^a	35.3 ± 9.71 ^a	151 ± 5.77 ^a	46.1 ± 5.07 ^a	43.6 ± 0.43 ^b	5.63 ± 2.77 ^a	33.6 ± 6.28 ^a	5.10 ± 3.54 ^a	9.84 ± 4.64 ^a
	435 ± 22.4 ^c	109.5 ± 12.1 ^c	600 ± 54.7 ^c	83.3 ± 2.01 ^b	96.6 ± 0.26 ^d	15.0 ± 5.22 ^b	65.0 ± 3.48 ^b	52.4 ± 4.63 ^d	33.7 ± 7.42 ^{bc}
EtOAc	152 ± 2.41 ^b	126 ± 13.0 ^c	381 ± 10.5 ^b	44.8 ± 8.55 ^a	81.9 ± 0.89 ^c	10.0 ± 0.68 ^{ab}	35.9 ± 3.68 ^a	34.1 ± 3.14 ^c	13.8 ± 5.16 ^a
MeOH									

Water	55.9 ± 1.75 ^a	68.9 ± 3.32 ^b	179 ± 11.9 ^a	36.1 ± 1.56 ^a	23.1 ± 1.10 ^a	22.2 ± 1.21 ^c	28.0 ± 4.65 ^a	17.6 ± 5.18 ^b	25.7 ± 4.09 ^{ab}
Asc	N/A	N/A	879 ± 12.0 ^d	45.3 ± 5.03 ^a	97.9 ± 0.06 ^{de}	4.41 ± 1.82 ^a	ND	N/A	N/A
Trolox	N/A	N/A	657 ± 16.9 ^c	91.9 ± 3.49 ^b	99.3 ± 1.10 ^e	13.5 ± 2.05 ^b	84.1 ± 5.12 ^c	N/A	N/A
Aca	N/A	N/A	N/A	N/A	N/A	N/A	N/A	88.3 ± 1.31 ^e	43.6 ± 11.1 ^c

“AAI”, α -amylase inhibitory activity; “ABTS”, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; “Aca”, acarbose; “AGI”, α -glucosidase inhibitory activity; “AnLP”, anti-linoleic acid peroxidation activity; “Asc”, ascorbic acid; “DCM”, dichloromethane extract; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; “FRAP”, Fe³⁺ reducing antioxidant power; “EtOAc”, ethyl acetate extract; “GAE”, gallic acid equivalent; “MeOH”, methanol extract; “N/A”, not applicable; “ND”, not determined; “NO”, nitric oxide radical scavenging activity; “QE”, quercetin equivalent; “TFC”, total flavonoid content; “TPC”, total phenol content. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Compared to the other extracts, the EtOAc extract had the highest ($p < 0.05$) α -glucosidase and α -amylase inhibitory activities (**Table 5.1**). The MeOH had moderate α -glucosidase inhibitory activity. The EtOAc and MeOH extracts showed a stronger inhibitory activity on α -glucosidase than α -amylase, which is consistent with a recent report, where litchi peel hydroalcoholic extracts showed more potent inhibition on α -glucosidase than α -amylase (Tanaka *et al.*, 2024). The enzyme inhibitory effect of the polar extracts of litchi peel has been linked to the presence of notable flavonoids such as quercetin glycosides, proanthocyanidin, catechins and derivatives, etc. (Tanaka *et al.*, 2024), which are also known to exert enzyme inhibitory action (Dirir *et al.*, 2022). α -Glucosidase inhibitors exert glycaemic control by reducing postprandial blood glucose (Kashtoh *et al.*, 2022), reiterating the anti-hyperglycaemic potential of litchi peel.

5.4.2 Phytochemical and bioactive profiles of the fractions and sub-fractions from the ethyl acetate peel extract

Gradient column chromatography yielded fractions that were pooled into 8 major fractions (fractions 1 – 8) based on their TLC profiles (**Table S2 of Appendix**). Although fraction 5 had the lowest total phenol content, its flavonoid content was significantly ($p < 0.05$) higher than other fractions (**Table 5.2**), which suggests that this fraction may contain some of the key bioactive flavonoids reported in litchi peel (Zhao *et al.*, 2006; Zhao *et al.*, 2007; Li *et al.*, 2007; Tanaka *et al.*, 2024).

Table 5.2: Total phenol and flavonoid contents and *in vitro* Fe³⁺ reducing, radical scavenging and enzyme inhibitory activities of fractions from the crude ethyl acetate extract at 35 µg/mL.

Fractions and standards	TPC (mg/g GAE)	TFC (mg/g QE)	FRAP (mg/g GAE)	DPPH (%)	ABTS (%)	AGI (%)	AAI (%)
F1	361 ±	42.2 ±	167 ±	40.7 ±	80.6 ±	22.0 ±	14.1 ±
	66.0 ^{de}	8.43 ^b	3.08 ^d	6.98 ^{bc}	1.42 ^a	4.44 ^a	4.98 ^{ab}
F2	322 ±	71.7 ±	166 ±	34.7 ±	80.1 ±	37.7 ±	15.1 ±
	55.2 ^d	5.65 ^c	3.12 ^d	4.67 ^{bc}	1.73 ^a	5.31 ^{bc}	3.19 ^{ab}
F3	220 ±	73.5 ±	124 ±	14.9 ±	77.9 ±	49.3 ±	22.9 ±
	19.5 ^{bc}	8.55 ^c	16.5 ^b	1.30 ^a	6.00 ^a	8.13 ^{cd}	9.33 ^b
F4	195 ±	70.8 ±	78.3 ±	27.8 ±	97.6 ±	37.5 ±	7.20 ±
	19.7 ^{ab}	16.0 ^c	12.2 ^a	6.27 ^{ab}	2.22 ^d	5.98 ^{bc}	3.57 ^a
F5	133 ±	104 ±	122 ±	28.3 ±	98.2 ±	68.1 ±	36.7 ±
	25.8 ^a	6.11 ^d	6.70 ^b	5.14 ^b	1.17 ^d	1.51 ^e	4.86 ^c
F6	287 ±	54.1 ±	166 ±	39.4 ±	98.0 ±	31.7 ±	6.39 ±
	4.33 ^{cd}	7.50 ^{bc}	1.36 ^d	8.08 ^{bc}	0.06 ^d	7.40 ^{ab}	1.82 ^a
F7	422 ±	18.9 ±	179 ±	43.1 ±	97.4 ±	18.4 ±	14.6 ±
	3.88 ^e	2.93 ^a	2.05 ^{de}	1.05 ^c	2.21 ^d	1.87 ^a	2.30 ^{ab}
F8	186 ±	18.2 ±	145 ±	38.2 ±	96.9 ±	21.9 ±	10.8 ±
	16.4 ^{ab}	2.12 ^a	4.87 ^c	0.73 ^{bc}	1.08 ^{cd}	5.52 ^a	3.74 ^{ab}
Asc	N/A	N/A	290 ±	41.4 ±	90.3 ±	N/A	N/A
			4.36 ^f	1.89 ^c	1.88 ^{bc}		
Trolox	N/A	N/A	192 ±	79.0 ±	87.0 ±	N/A	N/A
			3.93 ^e	4.13 ^d	1.50 ^{ab}		
Aca	N/A	N/A	N/A	N/A	N/A	65.8 ±	39.9 ±
						2.76 ^e	3.45 ^c

“AAI”, α-amylase inhibitory activity; “ABTS”, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; “Aca”, acarbose; “AGI”, α-glucosidase inhibitory activity; “Asc”, ascorbic acid; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; “FRAP”, Fe³⁺ reducing antioxidant power; “GAE”, gallic acid equivalent; “N/A”, not applicable; “QE”, quercetin equivalent; “TFC”, total flavonoid content; “TPC”, total phenol content. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

In fact, LC-MS analysis of fraction 5 showed the notable presence of epicatechin, among other flavonoids (Table 5.3 and Figure 5.2), which may have influenced its more robust ($p < 0.05$) α-glucosidase and α-amylase inhibitory activity relative to the other fractions (Table 5.2). Moreover, the enzyme inhibitory activity of epicatechin and derivatives has been documented (Wu *et al.*, 2019; Xu *et*

al., 2019; Şöhretoğlu *et al.*, 2023). Fraction 5 also showed appreciable ABTS radical scavenging activity (Table 5.2) and thus was selected for further fractionation and isolation of bioactive flavonoids.

Table 5.3: LC-MS data of fraction 5 from the ethyl acetate extract of litchi peel.

Identified Compounds	Ontology	ART (min)	AMz	Total score	Peak height	Conc. (mg/L)
Procyanidin B5	Biflavonoids and polyflavonoids	8.241	577.13507	7.6791	1,280	0.3
Pseudolaroside B;(-)- Pseudolaroside B	Hydrolyzable tannins	8.802	329.0878	7.4069	2,855	0.7
Butyl 3-O-caffeoylquininate	Quinic acids and derivatives	9.147	409.15076	7.3007	3,121	0.8
Glucosyringic acid;Syringin 4-O-beta-glucoside	Hydrolyzable tannins	9.722	359.09879	7.349	5,648	1.4
Epicatechin	Catechins	12.133	289.07214	8.0017	370,407	94.5
Kievitone hydrate	8-prenylated isoflavanones	13.707	373.13028	4.7465	14,903	3.8
Garcimangosone D	Phenolic glycosides	14.2	391.1037	7.3375	4,686	1.2
5-hydroxy-2-(4-hydroxyphenyl)-7-[[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-3,4-dihydro-2H-1-benzopyran-4-one	Flavonoid-7-O-glycosides	15.163	433.11365	7.5754	1,137	0.3
Garcimangosone D	Phenolic glycosides	15.254	391.1037	7.0551	1,280	0.3
Polydine	Flavonoid-7-O-glycosides	16.175	421.11395	7.3603	4,486	1.1
Isolariciresinol 9'-O-beta-D-glucoside	Lignan glycosides	16,435	521.20264	7.1728	7,835	2.0
Quercetin 3-galactoside	Flavonoid-3-O-glycosides	16.659	463.0878	7.7967	16,029	4.1
Aspalathin	2'-Hydroxy-dihydrochalcones	16.834	451.12402	7.1687	7,039	1.8
Aurasperone C	Naphthopyranones	16.843	591.15076	7.5058	27,229	6.9
Isolariciresinol 9'-O-beta-D-glucoside	Lignan glycosides	16.97	521.20251	6.0419	25,518	6.5

Identified Compounds	Ontology	ART (min)	AMz	Total score	Peak height	Conc. (mg/L)
Aurovertin G	C-glycosyl compounds	17.308	491.19247	6.3281	50,720	12.9
Hovenitin I	Epigallocatechins	17.492	333.06146	8.0014	9,169	2.3
Avicularin	Flavonoid-3-O- glycosides	17.611	433.07831	6.7129	46,840	11.9
Quercitrin	Flavonoid-3-O- glycosides	18.322	447.09387	7.9544	46,688	11.9
Ethyl 7-epi-12- hydroxyjasmonate glucoside	Fatty acyl glycosides of mono- and disaccharides	18.513	415.19803	6.1113	294	0.1
Kaempferol 3- α -L- arabinopyranoside	Flavonoid glycoside	18.969	417.08301	7.5182	11,122	2.8
Phlorizin	Polyphenol glycoside	19.097	435.1297	7.9116	24,326	6.2

Abbreviations: “ART”, average retention time”; “AMz”, average mass-to-charge ratio; “Conc.”, concentration.

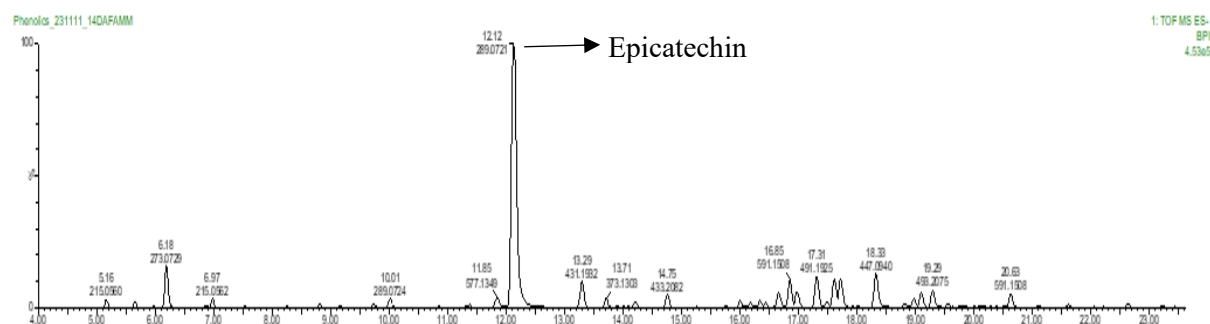


Figure 5.1: LC-MS chromatogram of fraction 5 from the ethyl acetate extract of litchi peel.

Subjecting fraction 5 to Sephadex chromatography yielded 7 sub-fractions, which were assessed for their radical scavenging, anti-lipid peroxidative, α -glucosidase inhibitory, and cellular glucose uptake activities. The anti-lipid peroxidative and α -glucosidase inhibitory activities of sub-fractions 4 and 5 significantly ($p < 0.05$) outperformed the activities of the other sub-fractions (**Table 5.4**). In fact, at a tested concentration of 20 $\mu\text{g/mL}$, their anti-lipid peroxidative and α -glucosidase activities were comparable to that of Trolox and acarbose, respectively. The data suggests that the epicatechin principle in fraction 5 (**Table 5.3** and **Figure 5.1**) may have been eluted in sub-fractions 4 and 5, which influenced their potent activities. Moreover, the protective effect of epicatechin and derivatives on lipid

peroxidative damage (Terao *et al.*, 1994; Shaki *et al.*, 2017), as well as their α -glucosidase inhibitory effects (Wu *et al.*, 2019; Xu *et al.*, 2019; Şöhretoğlu *et al.*, 2023) have been documented.

Table 5.4: *In vitro* radical scavenging, anti-linoleic acid peroxidative, enzyme inhibitory and glucose uptake modulatory activities of sub-fractions at 20 $\mu\text{g/mL}$.

Sub-fractions and standards	DPPH (%)	ABTS (%)	AnLP (%)	AGI (%)	GUMA (%)
SBF1	37.7 $\pm$ 4.26 ^{cd}	84.8 $\pm$ 3.96 ^{bc}	25.9 $\pm$ 7.00 ^{ab}	22.4 $\pm$ 2.69 ^a	NAD
SBF2	26.7 $\pm$ 6.24 ^{abc}	72.8 $\pm$ 1.34 ^a	19.9 $\pm$ 2.21 ^a	36.4 $\pm$ 4.03 ^{ab}	NAD
SBF3	22.1 $\pm$ 3.81 ^{ab}	72.1 $\pm$ 4.63 ^a	38.7 $\pm$ 5.52 ^b	46.8 $\pm$ 12.7 ^{bc}	14.0 $\pm$ 6.92 ^a
SBF4	32.2 $\pm$ 6.36 ^{bcd}	86.8 $\pm$ 2.54 ^c	65.5 $\pm$ 8.95 ^c	65.8 $\pm$ 2.85 ^d	38.2 $\pm$ 5.81 ^c
SBF5	26.1 $\pm$ 1.99 ^{abc}	87.1 $\pm$ 0.90 ^c	60.1 $\pm$ 3.72 ^c	56.0 $\pm$ 3.00 ^{cd}	28.0 $\pm$ 2.47 ^b
SBF6	18.2 $\pm$ 4.00 ^a	91.8 $\pm$ 4.31 ^c	29.6 $\pm$ 7.80 ^{ab}	29.2 $\pm$ 6.00 ^a	NAD
SBF7	15.3 $\pm$ 4.89 ^a	86.0 $\pm$ 1.71 ^c	23.4 $\pm$ 6.95 ^{ab}	23.9 $\pm$ 5.22 ^a	11.7 $\pm$ 3.28 ^a
Asc	40.0 $\pm$ 2.06 ^d	75.9 $\pm$ 2.77 ^{ab}	ND	N/A	N/A
Trolox	68.2 $\pm$ 3.87 ^e	76.6 $\pm$ 1.83 ^{ab}	70.7 $\pm$ 4.05 ^c	N/A	N/A
Aca	N/A	N/A	N/A	58.8 $\pm$ 4.04 ^{cd}	N/A

“ABTS”, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; “Aca”, acarbose; “AGI”, α -glucosidase inhibitory activity; “AnLP”, anti-linoleic acid peroxidation activity; “Asc”, ascorbic acid; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; “GUMA”, glucose uptake modulatory activity; “N/A”, not applicable; “ND”, not determined. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Furthermore, sub-fractions 4 and 5 showed stronger ($p < 0.05$) glucose uptake modulatory activities, relative to the other sub-fractions in Chang cells (**Table 5.4**). Although, sub-fraction 4 was not as potent as sub-fraction 5 ($p < 0.05$). Cellular glucose uptake is a key mechanism in physiological glucose homeostasis, which is impaired during T2D due to insulin resistance (Galicia-Garcia *et al.*, 2020). Thus, the potent glucose uptake modulatory activity of sub-fraction 4 suggests it contains flavonoid(s) that can potentiate glycaemic control via improving insulin signalling and cellular glucose uptake and utilization. Epicatechin was the most plausible influencing bioactive flavonoids of sub-fraction 4, since it was the most abundant flavonoid in the parent fraction (fraction 5) (**Table 5.3** and **Figure 5.2**). It is a dietary supplement reported to improve cellular insulin sensitivity and glucose tolerance in obese and T2D individuals (Cremonini *et al.*, 2019). Hence, sub-fraction 4 was probed further to isolate bioactive epicatechin.

5.4.3 Spectrometric and bioactive profiles of the isolated compound

Epicatechin was isolated from sub-fraction 4 using preparative TLC as a light/pale yellow powder. The spectral data of epicatechin (**Figure 5.2**) is as follows: ESI-MS⁺: m/z 291 [M + H]⁺, 313 [M + Na]⁺, 387 [M + K]⁺ (**Figure S1 of Appendix**); ESI-MS⁻: m/z 289 [M - H]⁻, 325 [M + Cl]⁻, 579 [2M - H]⁻ (**Figure S2 of Appendix**). ¹H-NMR spectrum (400 MHz, CD₃OD) as shown in **Table 5.5**: δ_H 6.99 (1H, s, H-2'), δ_H 6.80 (1H, dd, J = 8.2 Hz, H-6'), δ_H 6.78 (1H, d, 8.1 Hz, H-5'), δ_H 5.96 (1H, d, H-6), δ_H 5.94 (1H, d, H-8), δ_H 4.84 (1H, brs, H-2), δ_H 4.19 (1H, s, H-3), δ_H 2.88 (1H, dd, J = 4.3, 16.8 Hz, H-4_{axi}) and δ_H 2.75 (1H, dd, H-4_{eq}), see Table 1. ¹³C-NMR (100 MHz, CD₃OD): δ_C 156.6 (C-5), 156.3 (C-8a), 156.0 (C-7), 144.6 (C-3'), 144.4 (C-4'), 130.9 (C-1'), 113.9 (C-2'), 118.0 (C-6'), 114.5 (C-5'), 98.7 (C-4a), 95.0 (C-6), 94.5 (C-8), 78.5 (C-2), 66.1 (C-3) and 27.9 (C-4).

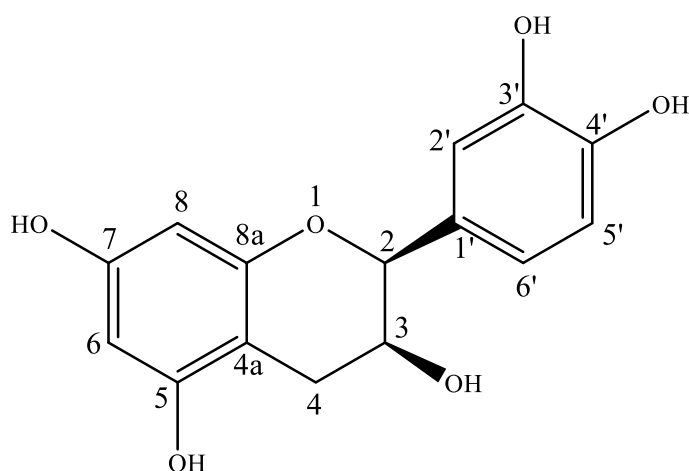


Figure 5.2: Structure of (+)-*Epi*-catechin.

The ¹H-NMR (400 MHz) spectrum of the compound showed four groups of chemical shift values attributable to the flavonoid's skeleton (Tajuddeen *et al.*, 2016). The first group at δ_H 6.99 (1H, s, H-2'), δ_H 6.80 (1H, dd, J = 8.2 Hz, H-6') and δ_H 6.78 (1H, d, 8.1 Hz, H-5') forming an ABX system (trisubstituted benzene ring-B of flavonoids); the second group at δ_H 5.96 (1H, d, H-6) and δ_H 5.94 (1H, d, H-8) corresponding to an AX system (tetrasubstituted benzene ring-A of flavonoids); the third and fourth groups formed by two oxymethine protons at δ_H 4.84 (1H, brs, H-2) and δ_H 4.19 (1H, s, H-3); and two methylene anisotropic protons at δ_H 2.88 (1H, dd, J = 4.3, 16.8 Hz, H-4_{axi}) and δ_H 2.75 (1H, dd, H-4_{eq}) (**Figure S3 of Appendix**) attributable to proton 2, 3, and 4 of saturated ring C of flavan-3-ol type flavonoid, respectively (Jung *et al.*, 2012, Tajuddeen *et al.*, 2016). The ¹³C-NMR (100 MHz, CD₃OD) spectrum indicated the presence of 15 carbon atoms [five oxygenated aromatic carbons at δ_C 156.6 (C-5), 156.3 (C-8a), 156.0 (C-7), 144.6 (C-3') and 144.4 (C-4'); five aromatic methine carbons at 113.9 (C-2'), 118.0 (C-6'), 114.5 (C-5'), 95.0 (C-6) and 94.5 (C-8); two aromatic quaternary carbons at 130.9 (C-1') and 98.7 (C-4a); two aliphatic oxygenated methine carbons at 78.5 (C-2) and 66.1 (C-3), and one aliphatic methylene carbon at 27.9 (C-4)] (**Figure S4 of Appendix**). The DEPT 135

spectrum confirms the presence of one methylene and seven methine carbons. All the above above-mentioned information confirms compound 1 to be a flavan-3-ol flavonoid type (Abdullahi *et al.*, 2017). The HSQC, HMBC, COSY and NOESY spectra (**Figure S5 – S8 of Appendix**) the structure of the compound was a Catechin epimer. The presence of signals of asymmetric carbons around δ 79 (C-2) and 67 (C-3) shows the compound is an *Epi*-catechin rather than a Catechin [signals around δ 82 (C-2) and 68 (C-3)]. The chemical shift values of protons H-2 and H-3 around δ 4.85 and 4.18 confirms the compound is a (+)-*Epi*-catechin (**Figure 5.2**) (El-razek ABD, 2007), which has been previously isolated from *Euphorbia thymifolia* Linn. and characterized (Kainsa *et al.*, 2016) as shown in **Table 5.5**.

Table 5.5: ^1H (400 MHz) and ^{13}C (100 MHz) NMR data of the isolated compound compared with reported literature.

Position	Compound 1		Reported literature (El-Razek, 2007)	
	δ ^1H (J in Hz)	δ ^{13}C	δ ^1H (J in Hz)	δ ^{13}C
2	4.84 (s)	78.5	4.85 (s)	79.6
3	4.19 (s)	66.1	4.18 (br s)	67.1
4axi	2.88 (dd, J = 4.3; 16.8 Hz)	27.9	2.83 (br d, 17.0 Hz)	29.2
4eq	2.75 (dd, J = 4.3; 16.8 Hz)	27.9	2.70 (br d, 17.0 Hz)	29.2
4a		98.7		100.0
5		156.6		157.8
6	5.96 (d)	95.0	5.99 (d, J = 1.8 Hz)	96.3
7	5.94 (d)	156.0	5.88 (d, J = 1.8 Hz)	157.8
8		94.5		95.5
8a		156.3		157.4
1'		130.9		132.5
2'	6.99 (s)	113.9	7.01 (d, 1.8 Hz)	115.5
3'		144.6		145.6
4'		144.4		145.5
5'	6.78 (d, J = 8.1 Hz)	114.5	6.75 (d, J = 8.1 Hz)	115.7
6'	6.80 (dd, J = 8.1; 2.2 Hz)	118.0	6.80 (dd, J = 8.0; 1.8 Hz)	119.6

Bioactivity assessment of the isolated epicatechin showed that it demonstrated a dose-dependent inhibitory action (IC_{50} = 18.9 μM) on LPS-induced ROS production in RAW 264.7 macrophages, which was statistically comparable ($p > 0.05$) to that of ascorbic acid (IC_{50} = 9.57 μM) (**Table 5.6** and **Figure 5.3**), reiterating the antioxidant potency of epicatechin. LPS-induced ROS production has been linked to pro-inflammatory signalling, which can disrupt cellular processes and cause oxidative tissue injuries

due to excessive production of superoxide ions and hydrogen peroxide (Hsu *et al.*, 2002). Hence, it is plausible that the inhibitory effect of epicatechin on LPS-induced ROS production in RAW 264.7 macrophages was linked to both antioxidant and anti-inflammatory capacities. Moreover, the inhibitory effect of epicatechin on LPS-induced production of proinflammatory mediators and cytokines in RAW 264.7 macrophages has been reported (Wang *et al.*, 2014).

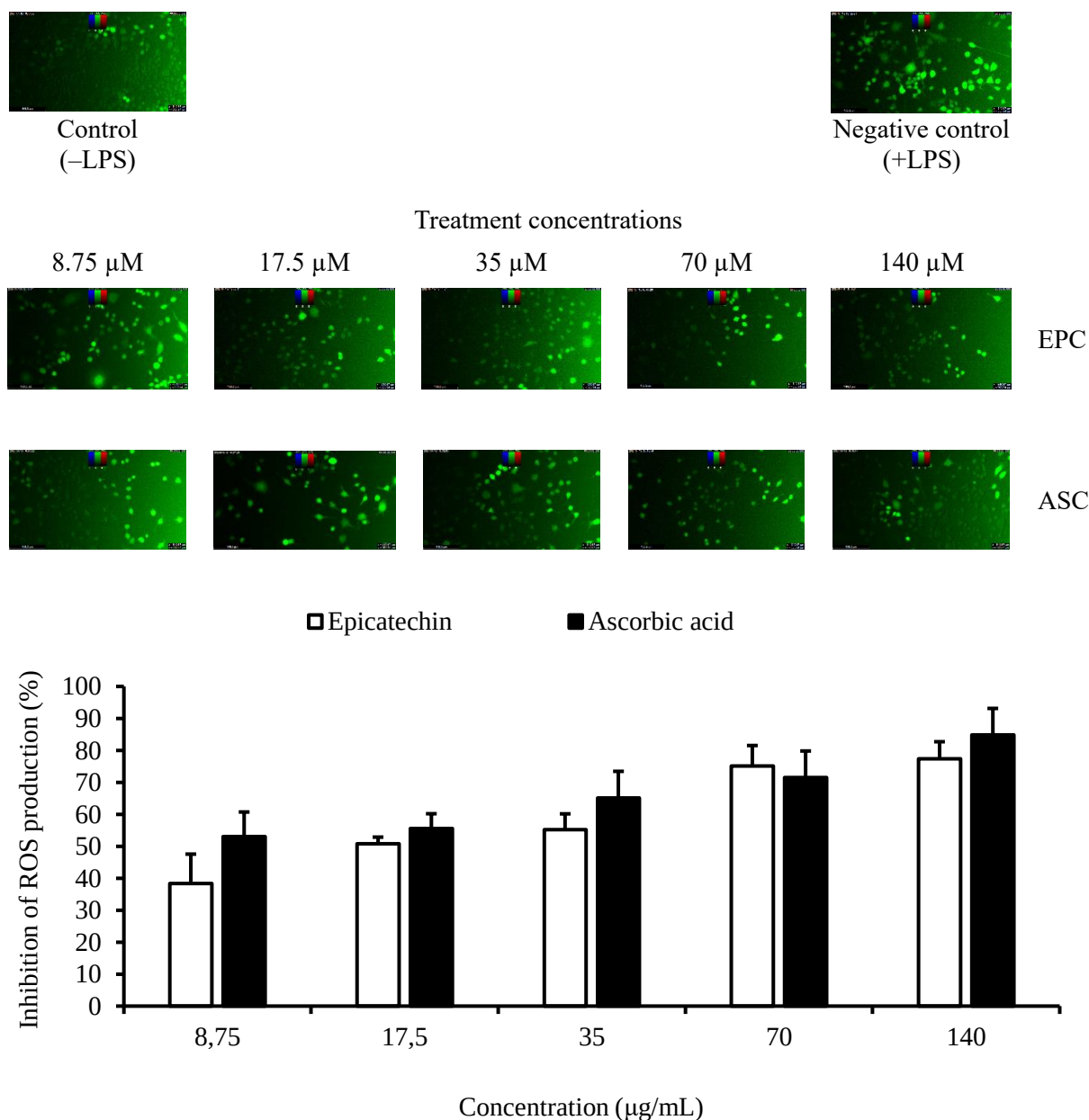


Figure 5.3: Dose-dependent inhibitory effect of epicatechin (EPC) and ascorbic acid (ASC) on ROS production in LPS-treated RAW 264.7 macrophages. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups at a given concentration when there are no similar letters. “LPS” means lipopolysaccharide.

Table 5.6: IC₅₀ and EC₅₀ values for the dose-dependent activities of the tested samples.

Assays	Epicatechin	Acarbose	Ascorbic acid
	IC ₅₀ and EC ₅₀ values (μM)		
α-Glucosidase inhibitory activity (IC ₅₀)	35.3 ± 5.75 ^a	11.0 ± 4.11 ^b	NA
Inhibition of ROS production in RAW 264.7 cells (IC ₅₀)	18.9 ± 5.44	NA	9.57 ± 4.10
Chang cell glucose uptake modulatory activity (EC ₅₀)	78.5 ± 13.6	NA	NA

“NA” means “not applicable”. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters attached to each data represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Furthermore, the isolated epicatechin demonstrated a dose-dependent glycaemic control potential. It had an inhibitory effect (IC₅₀ = 35.3 μM) on α-glucosidase (Table 5.6 and Figure 5.4), suggesting it could be a key flavonoid influencing the α-glucosidase inhibitory activity of the potent sub-fraction(s)/fraction(s) and extract of litchi fruit peel. In Chang cells, epicatechin exerted dose-dependent modulatory effect (EC₅₀ = 78.5 μM) on glucose uptake (Table 5.6 and Figure 5.6).

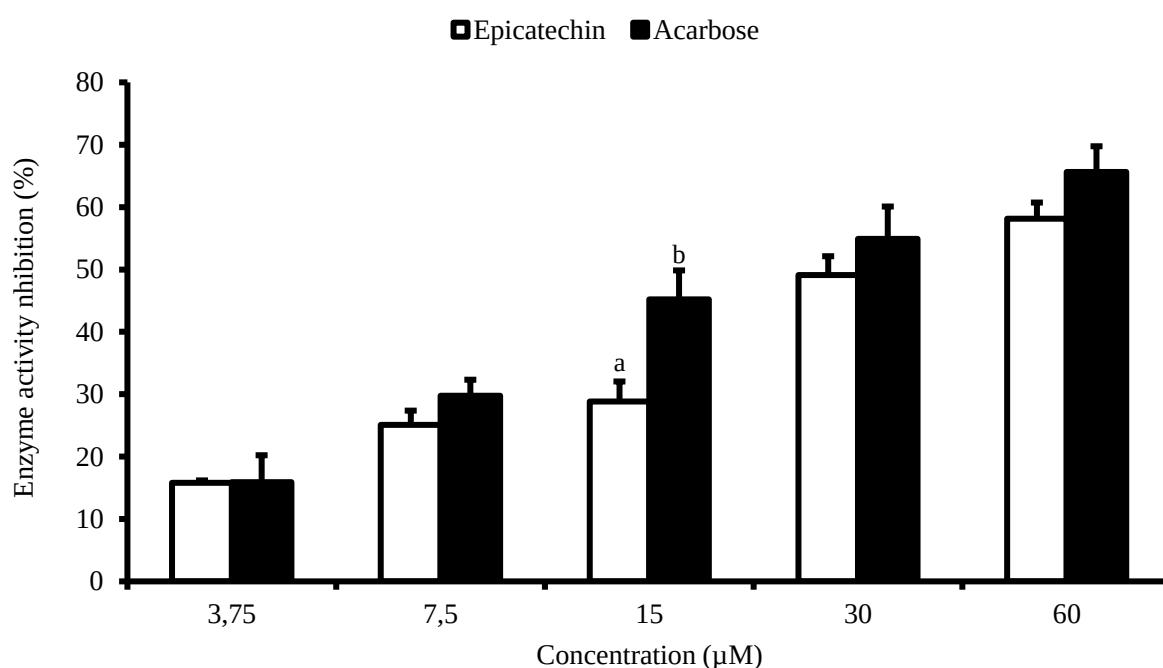


Figure 5.4: Dose-dependent inhibitory effect of epicatechin and acarbose on α-glucosidase activity. Data are presented as mean ± SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups at a given concentration when there are no similar letters.

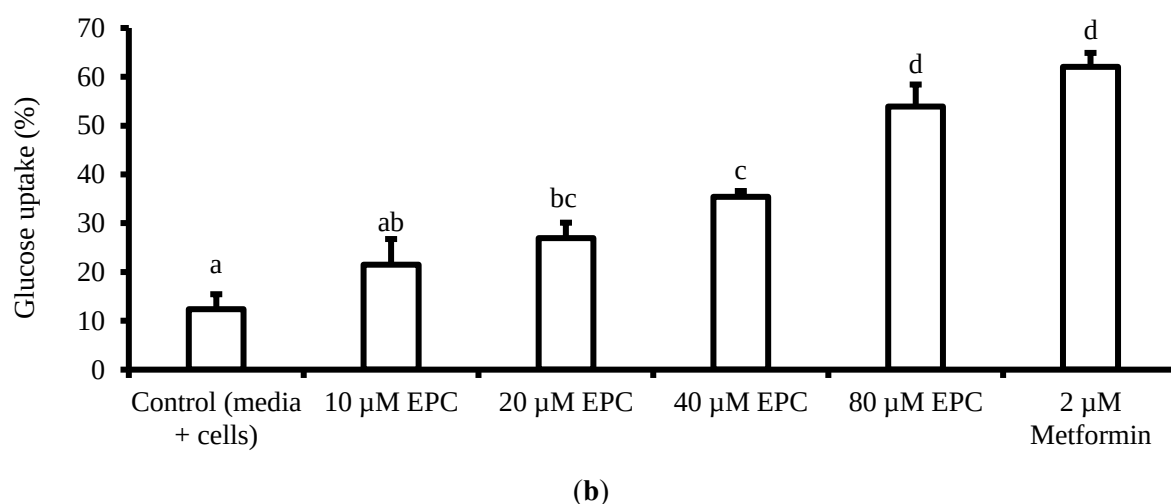
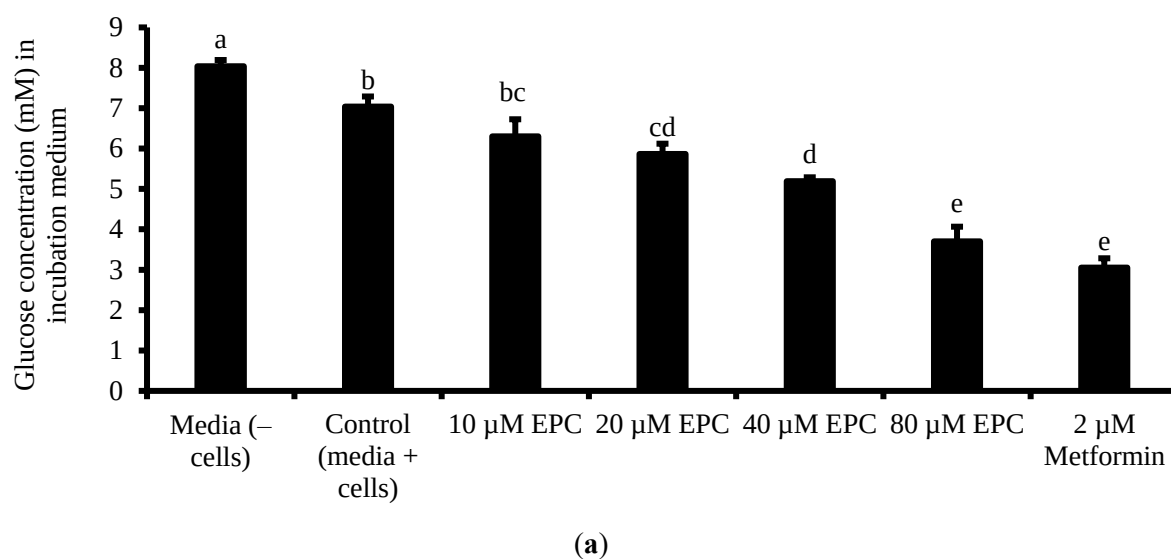


Figure 5.5: (a) Glucose concentration in the medium of cells receiving different treatments and (b) % glucose uptake of cells receiving different treatments. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between the treatments when there are no similar letters. “EPC” means epicatechin.

Relative to the untreated cells (control), an 80 μ M epicatechin concentration enhanced ($p < 0.05$) glucose uptake to an extent that was statistically comparable ($p > 0.05$) to the glucose uptake effect of a 2 μ M metformin, suggesting epicatechin may be effective in promoting cellular glucose uptake in cells of peripheral tissues, and may have influenced the glucose uptake activity of the potent sub-fractions 4 and 5 (Table 5.4). Documented evidence suggests that epicatechin may enhance glucose uptake by modulating insulin signalling via the PI3K/AKT signalling pathway and modulating the translocation of glucose transporters (Xu *et al.*, 2019; Chun *et al.*, 2022). Further studies are, however, needed to investigate this speculation.

5.5 Conclusion

In the present study, a bioassay-guided approach was used to probe into litchi fruit peel's antioxidant and anti-hyperglycaemic phytochemistry. Ethyl acetate solvent effectively extracted key flavonoids, which potentiated more potent antioxidant and enzyme inhibitory activities relative to the other solvent extraction. The flavonoid contents of fractions from the ethyl acetate extract also correlated with their α -glucosidase inhibitory activities. Epicatechin was the most abundant flavonoid in the fraction (fraction 5) with the highest inhibitory activity. Epicatechin was isolated from a sub-fraction (sub-fraction 4) of fraction 5. The isolated epicatechin demonstrated a dose-dependent inhibitory effect on α -glucosidase, modulatory effect on cellular glucose uptake, inhibitory effect on cellular ROS production, and possibly influenced the potent α -glucosidase inhibitory, glucose uptake modulatory, and anti-lipid peroxidative activity demonstrated by sub-fraction 4. The data of this study showed that litchi peels EtOAc extract may exert antioxidant and glycaemic control by alleviating ROS-mediated oxidative insults, inhibiting carbohydrate digesting enzymes, and modulating cell glucose uptake, and epicatechin may be a principal bioactive flavonoid influencing these antioxidant and glycaemic control properties. Thus, litchi peel EtOAc extract can be an alternative source to recover epicatechin with cellular glucose uptake modulatory and ROS production suppressive capacities.

CHAPTER 6

INVESTIGATING THE PHYTOCHEMISTRY AND UNDERLYING GLYCAEMIC CONTROL MECHANISMS OF *LITCHI CHINENSIS* SONN. (LITCHI) PEEL ETHYL ACETATE EXTRACT IN A FRUCTOSE/STREPTOZOTOCIN DIABETIC MODEL OF RATS

Prescript

This chapter investigated and reported the antioxidant and glycaemic control potential of litchi fruit peel ethyl acetate extract in a fructose/streptozotocin model of diabetic rats. The chapter has been published in **Nutrients** as follows: ***Izu GO, Mashele SS, Chukwuma CI*. (2024). Investigating the Phytochemistry and Underlying Glycemic Control Mechanisms of Litchi chinensis Sonn. (Litchi) Peel Ethyl Acetate Extract in a Fructose/Streptozotocin Diabetic Model of Rats. Nutrients, 16(21): 3644. <https://doi.org/10.3390/nu16213644>***. The above-mentioned publication was adopted in writing the content of this chapter.

6.1 Abstract

The glycaemic control potential and flavonoid profile of litchi have been documented for its hydroalcoholic extracts, while there is scarce information regarding its ethyl acetate extract. This study investigated the flavonoid profile, as well as the ameliorative potential and possible underlying mechanisms of litchi peel ethyl acetate extract on type 2 diabetes related pathologies in a fructose/streptozotocin (STZ) model of diabetic rats. Sprague Dawley rats were induced with diabetes by administering 10% fructose for 2 weeks and a single i.p. injection of low-dose (40 mg/kg bw) STZ. Thereafter, the animals were orally administered with a low-dose (150 mg/kg bw) and high-dose (300 mg/kg bw) of the peel extract (LDPE and HDPE, respectively) and metformin (200 mg/kg bw). Compared to untreated diabetic rats (AUC = 1004 mg.h/dL), the HDPE significantly ($p < 0.05$) improved glucose tolerance (AUC = 847 mg.h/dL), which was statistically comparable ($p > 0.05$) to the effect of metformin (AUC = 903 mg.h/dL). Serum insulin and pancreatic histology data showed that the STZ-induced pancreatic damage and insulin depletion was improved by the HDPE, which could be linked to the observed ameliorative effect of the extract on pancreatic lipid peroxidation and SOD and catalase activity. The extract further improved liver and muscle glycogen storage, as well as muscle hexokinase activity and Akt phosphorylation, suggesting that the extract exerts glycaemic control by enhancing glycogen storage and modulating insulin-mediated signaling of glucose uptake and utilization. LC-MS data and documented reports suggest that flavonoid, such as epicatechin, cinnamtannin B2, procyanidin B5 and proanthocyanidin A2 are the possible influencing compounds. The ethyl acetate extract of litchi peel could be a source of bioactive flavonoids that can potentiate glycaemic control in diabetes and mitigate the oxidative-stress related pathologies.

Keywords: *Antioxidant; ethyl acetate extract; flavonoids; fructose/streptozotocin diabetic rats; glycaemic control; Litchi fruit peel.*

6.2 Introduction

According to the International Diabetes Federation (IDF), the global prevalence of adults suffering from diabetes will increase from 537 million in 2021 to 783 million in 2045 (IDF, 2021), suggesting that the negative impact of diabetes on the global health and socioeconomic landscapes could worsen if precautionary measures are not implemented. Diabetes is characterized by detrimental metabolic imbalances caused by hormonal insufficiency or dysfunction, which results in persistent hyperglycaemia (Sanyaolu *et al.*, 2023). Type 2 diabetes (T2D) is the most prevalent type of diabetes, possibly due to its association with lifestyle related factors, such as physical inactivity, dieting and detrimental weight gain (Zhu *et al.*, 2021). These factors lead to insulin resistance, which is a key pathophysiological defect of T2D (Galicia-Garcia *et al.*, 2020; Zhu *et al.*, 2021). During insulin resistance, the homeostatic regulatory action of insulin on circulating glucose is impaired due to

dysfunctional signaling in the cells of peripheral tissues (Galicia-Garcia *et al.*, 2020). Cellular glucose uptake, nutrient metabolism and utilization are impaired, which lead to reduced glycaemic control and eventually persistent hyperglycaemia, especially if the function of the pancreatic β -cells has been concomitantly compromised (Galicia-Garcia *et al.*, 2020).

Persistent hyperglycaemia has been linked to the development of microvascular and macrovascular complications of diabetes and oxidative stress has been documented as a key underlying mediator (Bhatti *et al.*, 2022). Oxidative stress is caused by an imbalance between the physiological production of deleterious prooxidants or reactive oxygen species (ROS) and the antioxidant mechanisms to mop or quench the oxidant action of the prooxidants (Pizzino *et al.*, 2017). During diabetes, hyperglycaemia facilitates the production of ROS, which can oxidatively damage cellular macromolecules and organelles, while signaling associated inflammatory responses (Bhatti *et al.*, 2022). High blood glucose can cause glycation to key functional macromolecules, leading to production of advanced end products (AGEs), which are biological indicators of degenerative diseases, including diabetic complications (Bhatti *et al.*, 2022). Elevated lipid peroxidation in diabetes has also been documented, which can compromise cellular integrity due to damage of the lipid bilayer of cell membranes (Bhatti *et al.*, 2022). Also, deleterious radical byproducts of lipid peroxidation can also increase oxidative stress and cause oxidative damage to macromolecules (Bhatti *et al.*, 2022).

While there are many available commercial antidiabetic drugs, medicinal plants, including fruits and vegetables are increasingly been used as complementary and/or alternative therapies. Dietary antioxidant phenolic compounds present in fruits and vegetables offer a broad spectrum of pharmacological activities that could mitigate the development of diabetic complications by suppressing oxidative stress and inflammatory responses (Afam *et al.*, 2021). *Litchi chinensis* Sonn. (Sapindaceae) is a tropical and subtropical fruit with high commercial value due to its global consumption (Zhao *et al.*, 2020). The nutritional value and medicinal benefits of the fruit have also been documented (Zhao *et al.*, 2020). While studies have mentioned the antioxidant, anti-hyperglycaemic, anti-hypertensive, anti-obesogenic, anti-dyslipidaemic and hepatoprotective potentials of the fruit (Zhao *et al.*, 2020), documented evidence has shown that the non-edible peel could also be of medicinal relevance (Chukwuma *et al.*, 2021; Cano-Gómez *et al.*, 2024). Flavonoids, including catechins, procyanidins, proanthocyanidins, quercetin, rutin and their derivatives have been reported in the peel, which have been linked to some of the reported diabetes-related and antioxidant pharmacological properties of the peel (Zhao *et al.*, 2006; Chukwuma *et al.*, 2021; Cano-Gómez *et al.*, 2024). It has been shown that the fruit peel has a strong α -glucosidase inhibitory potential (Tanaka *et al.*, 2024), indicating it could potentiate glycaemic control by reducing postprandial blood glucose increase (Derosa and Maffioli, 2012). In high-fat-diet fed rodents, the peel powder and its procyanidin-rich fraction were shown to improve the profiles of blood lipids and glucose, and thus ameliorating diabetes-related metabolic alterations (Rong *et al.*, 2017; Queiroz *et al.*, 2018). In *db/db* mice, litchi peel ethanol extract

treatment has been shown to ameliorate type 2 diabetic pathologies, including elevated blood glucose, hepatic gluconeogenesis, oxidative stress and associated inflammatory responses (Yang *et al.*, 2024).

A review on the pharmacology potential of litchi fruit seed and peel (Chukwuma *et al.*, 2021) suggests that diabetes related pharmacological investigations have mostly been demonstrated in the hydro-alcoholic extracts or isolates with minimal investigations on other solvent extracts, such as the ethyl acetate extract. Ethyl acetate solvent extraction can cause hydrogen bonds formation with carboxyl and hydroxyl groups, resulting in the extraction of medium polar to polar phenolics and/or flavonoids (Prasad *et al.*, 2010), which could influence potent medicinal properties. In fact, documented qualitative evidence has shown that the ethyl acetate extract of litchi peel contains relatively similar proportion phenolic compounds and flavonoids as the hydroalcoholic extract (Chauhan *et al.*, 2021). A previous study has proven that litchi fruit ethyl acetate extract demonstrates potent immunomodulatory potential (Yang *et al.*, 2013). In a more recent antidiabetic study, a comparative assessment in alloxan-induced type 1 diabetic Wistar rats suggests that the blood glucose lowering, and antioxidant effect of litchi peel ethyl acetate extract outperformed that of the hydroalcoholic extract (Chauhan *et al.*, 2021), suggesting that litchi peel ethyl extract may possess ameliorative potential against T2D associated pathologies. However, this speculation, as well as the possible mechanisms remain elusive and warrants investigation. Therefore, this study investigated the ameliorative potential and possible underlying mechanisms of litchi peel ethyl acetate extract on T2D related pathologies in a fructose/low dose streptozotocin (STZ) model of diabetic rats.

6.3 Materials and methods

Please refer to **sub-sections 3.1, 3.6 and 3.7 of chapter 3** for the materials and methods of this chapter.

6.4 Results and discussion

The antidiabetic and antioxidant potential of litchi peel has been documented (Rong *et al.*, 2017; Queiroz *et al.*, 2018; Chukwuma *et al.*, 2021; Tanaka *et al.*, 2024; Yang *et al.*, 2024). However, study trends show that the hydroalcoholic extracts have mostly been studied, while the ethyl acetate extract, which has been shown to have promising antidiabetic, phenolic and flavonoid profiles (Chauhan *et al.*, 2021) remains under explored. The present study demonstrates the rich flavonoid profile of litchi peel ethyl acetate extract, as well as its ameliorative effects on T2D related pathologies in a fructose/STZ model of diabetic rats.

6.4.1 Phytochemical profile of litchi peel ethyl acetate extracts

The hydroalcoholic extracts of litchi peel have been extensively studied. Documented evidence has shown that the hydroalcoholic extracts of litchi peel are rich in flavonoids including catechins, proanthocyanidins and procyanidins, which are believed to be key bioactive flavonoids influencing the antioxidant, antidiabetic, anti-hypertensive and anti-inflammatory potentials of the hydroalcoholic peel extracts (Zhao *et al.*, 2006; Li and Jiang, 2007; Tanaka *et al.*, 2024; Yang *et al.*, 2024). On the other hand, a previous qualitative assessment (Chauhan *et al.*, 2021) has suggested that the ethyl acetate extract of litchi peel may also be rich in flavonoids as the hydroalcoholic extracts. However, specialized spectrometric profiling is still needed to confirm this. In our study, LC-MS analysis showed the notable presence of flavonoids, such as epicatechin, cinnamtannin B2, procyanidin B5 and proanthocyanidin A2 in the ethyl acetate extract of litchi peel (**Table 6.1 and Figure 6.1**). The presence of these flavonoids suggests that the ethyl acetate extract may also potentiate antioxidant and antidiabetic effects, which has been investigated and reported in this study.

Table 6.1: LC-MS data of litchi peel ethyl acetate extract.

Identified Compounds	Ontology	ART (min)	AMz	Total score	Peak height	Conc. (mg/kg)
Aralidioside	Iridoid O-glycosides	5.648	447.11478	6.9378	12,228	502
Cinnamtannin B2	Flavonoids	5.923	575.11932	7.0967	17,074	701
Procyanidin B5	Flavonoids	6.635	577.13574	8.5544	6,652	273
Epicatechin	Flavonoids	6.952	289.07196	8.1009	39,675	1,630
Epicatechin-(4beta->8)- epicatechin-(2beta->7,4beta->8)-catechin	Flavonoids	7.168	863.18451	7.0737	286,371	11,763
Aesculitannin G	Flavonoids	7.917	574.11267	5.7575	90,599	3,721
Proanthocyanidin A2	Flavonoids	8.306	575.12164	7.9145	110,862	4,554
Quercetin 3-galactoside	Flavonoid-3-O- glycosides	8.447	463.08835	6.7863	10,707	440
epi-12-Palmatoside G	Naphthopyranone glycosides	8.671	491.19287	5.8164	11,744	482
Astragalin 7-rhamnoside	Flavonoid-7-O- glycosides	8.82	593.1521	7.0845	16,335	671

“ART”, average retention time”; “AMz”, average mass-to-charge ratio; “Conc.”, concentration.

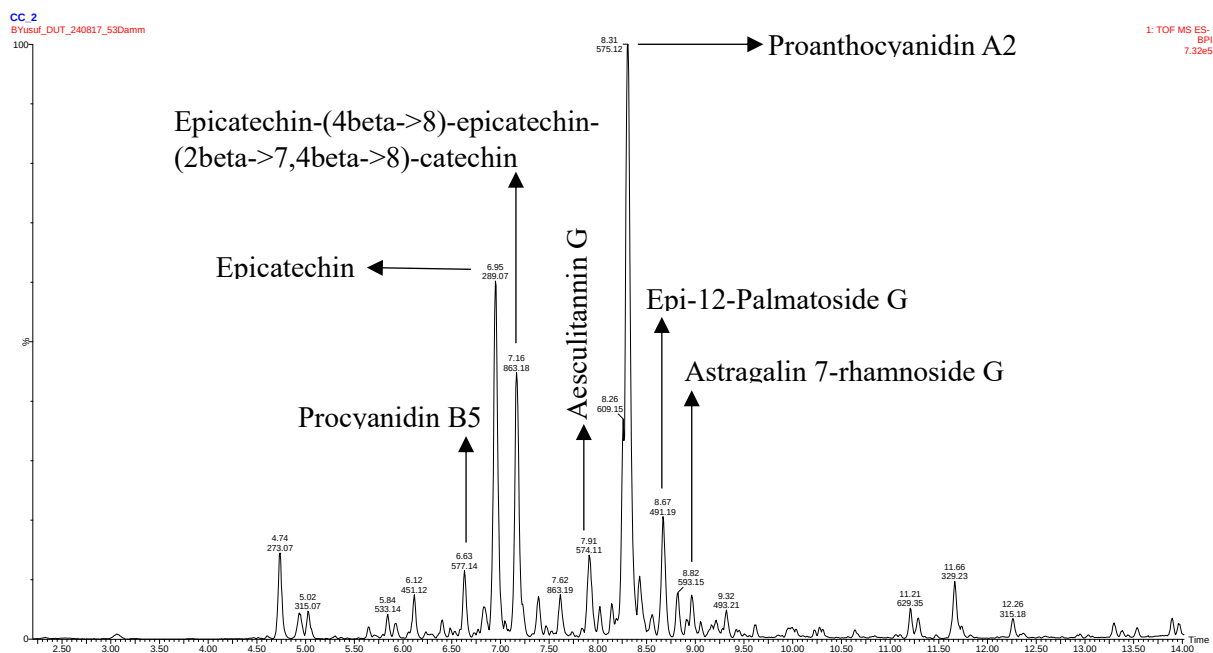


Figure 6.1: LC-MS chromatogram of litchi peel ethyl acetate extract.

6.4.2 Glycaemic control and antioxidant potential of litchi peel ethyl acetate

Persistent hyperglycaemia in diabetic state causes excessive urinary excretion of glucose, which is responsible for some of the classical symptoms of diabetes, including weight loss, excessive food consumption (polyphagia) and excessive fluid consumption (polydipsia) (Nigro *et al.*, 2018). In the present study diabetic rats significantly ($p < 0.05$) lost weight and consumed more food and water compared to the nondiabetic counter parts (**Table 6.2**), which is influenced by their higher ($p < 0.05$) blood glucose levels (**Figure 6.2**). Treatment with the peel extracts (LDPE and HDPE) and metformin did not significantly improve the diabetic weight loss or fluid intake. The HDPE, however, had appreciable positive effect on the diabetic weight loss and fluid intake particularly in the 4th week of intervention. In fact, although not normalized, the HDPE significantly ($p < 0.05$) reduced diabetic polyphagia (**Table 6.2**), suggesting a notable ameliorative effect of the litchi peel ethyl acetate extract, which can be further investigated long-term.

Table 6.2: Food intake, water intake and weekly body weight.

Groups	Body weight (g)						Food intake (g/rat/d)	Water intake (mL/rat/d)
	Week 0	Week 1	Week 2	Week 3	Week 3	Week 5		
NC	281 ±	307 ±	323 ±	324 ±	320 ±	328 ±	23.0 ±	47.7 ±
	9.97	15.3 ^b	17.7 ^b	18.8 ^b	19.5 ^b	20.4 ^b	1.25 ^c	8.52 ^b

DBC	294 ± 16.1	279 ± 24.2 ^a	271 ± 25.9 ^a	256 ± 23.9 ^a	252 ± 28.0 ^a	245 ± 20.8 ^a	46.3 ± 2.86 ^a	160 ± 30.8 ^a
LDPE	291 ± 23.6	279 ± 18.4 ^a	279 ± 26.3 ^a	268 ± 23.5 ^a	257 ± 25.3 ^a	254 ± 32.0 ^a	37.6 ± 8.13 ^b	159 ± 26.6 ^a
HDPE	306 ± 11.2	289 ± 18.6 ^a	282 ± 21.9 ^a	273 ± 28.3 ^a	265 ± 26.5 ^a	265 ± 28.6 ^a	35.2 ± 1.19 ^b	136 ± 3.61 ^a
MET	302 ± 17.3	285 ± 20.3 ^a	268 ± 23.6 ^a	261 ± 24.8 ^a	252 ± 25.8 ^a	259 ± 29.4 ^a	40.9 ± 5.66 ^{ab}	152 ± 5.87 ^a

“NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. Week 0 is the STZ injection; Week 1 is the diabetes confirmation; Week 2 to week 5 is the intervention period. For each parameter, statistical comparison was done among the groups. Significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

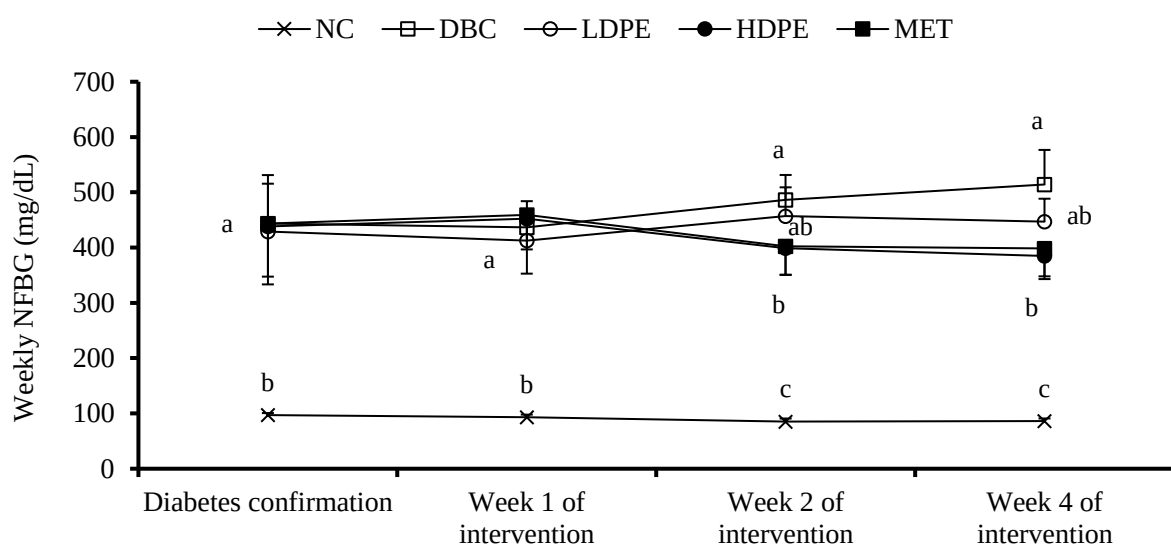
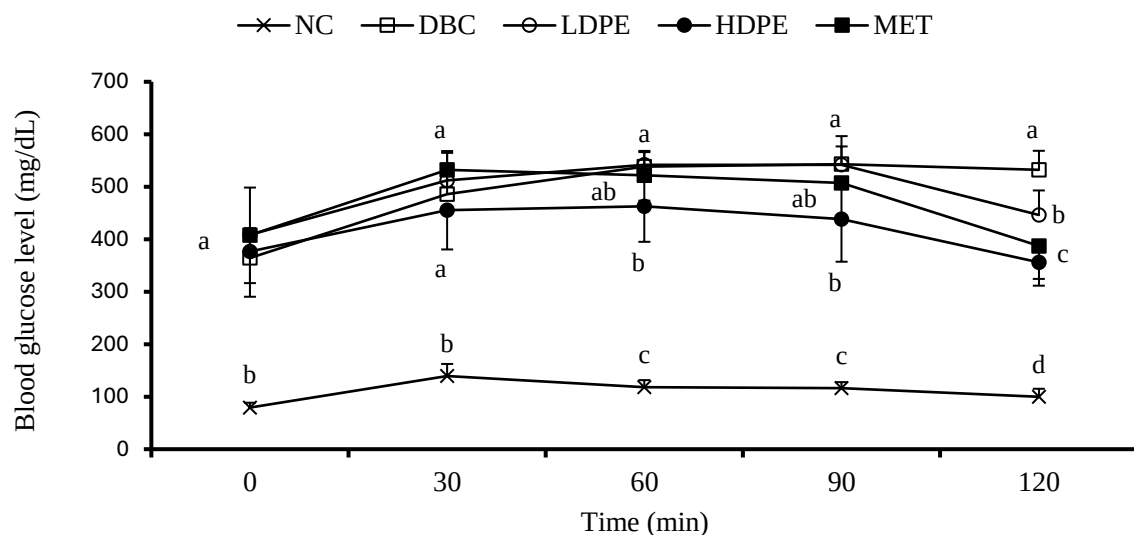
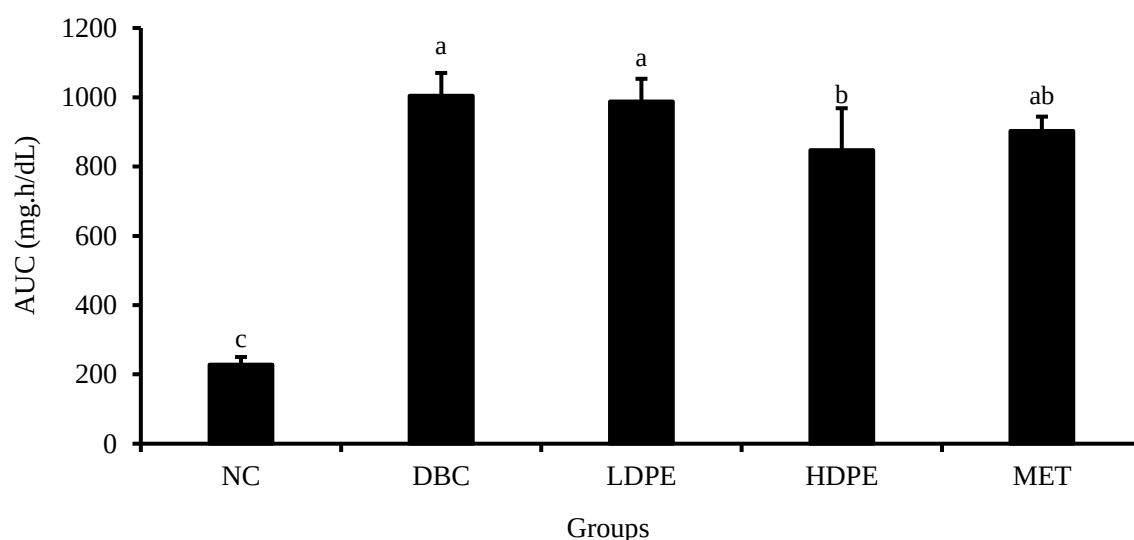


Figure 6.2: Weekly non fasting blood glucose (NFBG). “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each period, statistical comparison was done among the groups. Significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

Weekly NFBG data showed that the peel extract dose dependently reduced NFBG over the treatment period (**Figure 6.2**). Notably, the HDPE caused significant ($p < 0.05$) reduction in the 2nd and 4th week of the intervention period, which was statistically comparable to that of metformin treatment, suggesting the blood glucose lowering potential of the peel’s ethyl acetate extract. Furthermore, the extract also exhibited a dose dependent glucose tolerance effect (**Figure 6.3a**), with the effect of the HDPE being significant ($p < 0.05$) relative to the untreated diabetic animals (**Figure 6.3b**).



(a)



(b)

Figure 6.3: (a) Oral glucose tolerance data and **(b)** area under the curve. “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each period or parameter, statistical comparison was done among the groups. Significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

While the blood glucose lowering effect of litchi peel ethyl acetate extract has been previously documented, the effect was demonstrated in alloxan-induced diabetic rats (Chauhan *et al.*, 2021). The alloxan-induced and STZ-induced diabetic models are characterized by oxidative stress-mediated pancreatic β -cell damage and impaired insulin secretion (Lenzen, 2008), which suggests that the previously documented blood glucose lowering effect of litchi peel ethyl acetate extract (Chauhan *et al.*, 2021) may be influenced by an improvement of pancreatic β -cell damage, among other possible mechanisms. This is because supporting antioxidant data from the study by Chauhan *et al.* (2021)

showed that the ethyl acetate extract improved the pancreatic antioxidant status of the alloxan-induced diabetic rats.

Histology data comparing the pancreas of the normal rats (NC) with that of the untreated (DBC) and treated (LDPE, HDPE and MET) diabetic rats further reiterate the possible ameliorative effect of litchi peel ethyl acetate extract on chemical (STZ)-induced pancreatic damage (**Figure 6.4**).

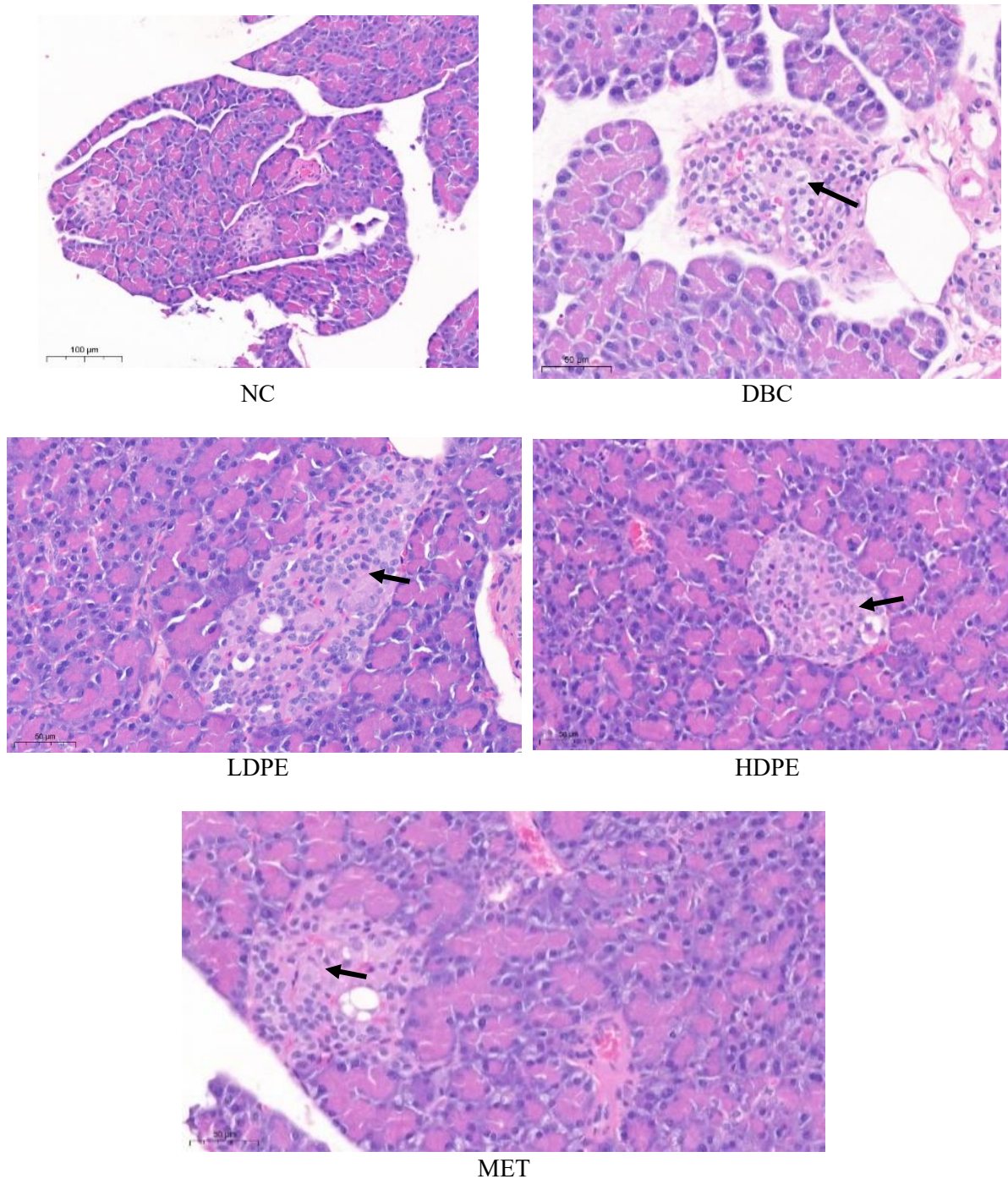


Figure 6.4: Histological images of the pancreatic tissues from rats. “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin.

The pancreatic histology of the normal rats (NC) showed that the exocrine pancreatic acinar cells appear normal and active. The available endocrine islets consisted of normal cells. The distribution pattern and size of the endocrine islets of Langerhans are considered normal with no sign of cellular or exudate accumulation (**Figure 6.4**).

The pancreatic histology of the untreated diabetic rats (DBC) showed features consistent with multifocal and mild pancreatic endocrine islet cell degeneration, which was caused by the low-dose (40 mg/kg bw) STZ treatment during induction (Lenzen, 2008). Typically, there were mild degenerative changes consisting of cytoplasmic vacuolization with mild swelling, mild lesions and possible atrophy within cells of the pancreatic islet (**Figure 6.4**). Similarly, the pancreatic histology of the rats treated with the peel extract and metformin (MET) showed features consistent with multifocal and mild pancreatic endocrine islet cell degeneration. However, the degeneration in the pancreatic islets of rats in HDPE group was minimal compared to that of the animals in the other groups (**Figure 6.4**), suggesting a somewhat ameliorate effect of the HDPE on STZ-induced pancreatic damage. This ameliorative effect further reflects the ability of the HDPE to significantly ($p < 0.05$) improve insulin secretion in diabetic rats as shown by the significantly ($p < 0.05$) higher serum insulin level compared to the untreated diabetic rats (**Table 6.3**).

Table 6.3: Serum insulin, tissue glycogen content and muscle tissue hexokinase activity.

Groups	Serum insulin level ($\mu\text{IU/mL}$)	Glycogen content (mg/g tissue)		Muscle hexokinase activity (nmol/min/mg protein)
		Liver	Muscle	
NC	2.43 ± 0.31^b	17.3 ± 3.56^c	1.35 ± 0.23^b	19.0 ± 5.33^b
DBC	1.16 ± 0.36^a	9.61 ± 1.19^a	0.88 ± 0.08^a	10.2 ± 1.68^a
LDPE	1.50 ± 0.16^a	12.4 ± 4.65^{ab}	0.95 ± 0.19^{ab}	11.8 ± 3.58^a
HDPE	2.17 ± 0.51^b	14.6 ± 2.40^{abc}	1.10 ± 0.20^{ab}	15.3 ± 2.00^{ab}
MET	1.12 ± 0.11^a	16.2 ± 4.68^c	1.30 ± 0.15^b	16.5 ± 5.07^{ab}

“NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each parameter, statistical comparison was done among the groups. Significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

It is plausible that the ameliorative effect of the HDPE on STZ-induced pancreatic damage may be linked to their pancreatic antioxidant capacities. The data of this study showed that the HDPE treatment significantly ($p < 0.05$) reduced pancreatic lipid peroxidation and concomitantly increased ($p < 0.05$) systemic and pancreatic catalase and SOD activities (**Table 4**).

Table 6.4: Lipid peroxidation and antioxidant enzymes activity in the serum and tissue of rats.

Groups	TBARS level (μM MDS equivalent)			SOD activity (% inhibition rate/mg protein)			Catalase activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)		
	Serum	Liver	Pancreas	Serum	Liver	Pancreas	Serum	Liver	Pancreas
NC	140 $\pm$	78.0 $\pm$	123 $\pm$	90.1 $\pm$	79.4 $\pm$	72.2 $\pm$	45.8 $\pm$	126 $\pm$	47.3 $\pm$
	28.0 ^c	16.9 ^c	37.3 ^c	6.90 ^b	5.91 ^b	8.19 ^c	7.24 ^b	13.8	4.00 ^d
DBC	564 $\pm$	234 $\pm$	294 $\pm$	66.3 $\pm$	66.4 $\pm$	44.4 $\pm$	27.3 $\pm$	112 $\pm$	15.2 $\pm$
	143 ^a	23.0 ^a	70.7 ^a	4.09 ^a	6.28 ^a	3.89 ^a	5.77 ^a	24.4	4.67 ^a
LDPE	419 $\pm$	209 $\pm$	222 $\pm$	84.0 $\pm$	69.1 $\pm$	48.2 $\pm$	33.3 $\pm$	123 $\pm$	26.0 $\pm$
	42.4 ^{ab}	62.4 ^{ab}	53.6 ^{ab}	8.71 ^b	1.24 ^a	3.38 ^a	6.67 ^a	15.7	2.32 ^b
HDPE	305 $\pm$	146 $\pm$	210 $\pm$	85.8 $\pm$	74.7 $\pm$	57.5 $\pm$	35.4 $\pm$	131 $\pm$	38.2 $\pm$
	62.1 ^a	22.2 ^{bc}	20.2 ^b	5.95 ^b	4.25 ^{ab}	2.00 ^b	3.08 ^{ab}	16.6	3.85 ^c
MET	411 $\pm$	217 $\pm$	257 $\pm$	70.5 $\pm$	68.3 $\pm$	47.4 $\pm$	30.7 $\pm$	116 $\pm$	22.9 $\pm$
	117 ^b	71.5 ^{ab}	37.9 ^{ab}	6.59 ^a	7.80 ^a	4.46 ^a	9.03 ^a	8.42	8.35 ^{ab}

“NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each parameter, statistical comparison was done among the groups. Significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

Catalase and SOD are antioxidant enzymes. SOD catalyzes the reduction of deleterious superoxide ions to hydrogen peroxide, which is reduced to water by the catalytic action of catalase (Lee and Park, 2021). Hence, the significantly higher pancreatic catalase and SOD activity of rats in the HDPE group, relative to the untreated diabetic rats suggests that the HDPE ameliorated STZ-induced ROS damage in the pancreatic tissue by mopping deleterious ROS. The potent *in vivo* antioxidant activity of the HDPE may be linked to notable presence of bioactive flavonoids including catechins, proanthocyanidins and procyanidins (Table 6.1 and Figure 6.1). Moreover, the hydroxyl radical and superoxide anion scavenging activity of proanthocyanidin B4, proanthocyanidin B2 and epicatechin isolated from litchi peel has been reported (Zhao et al., 2006), while the modulatory effect of catechins and proanthocyanidins *in vivo* antioxidant status and antioxidant enzymes activity have been documented (Patanè *et al.*, 2023). Furthermore, the key bioactive flavonoids present in the peel extracts may also influence its modulatory effect on insulin secretion. This is because a study has shown that epicatechin, a major flavonoid in the peel extract (Table 6.1 and Figure 6.1), modulates glucose-stimulated insulin secretion in INS-1 cells (Yang and Chan, 2018).

Other than the improvement of pancreatic antioxidant capacity, histology and insulin secretion, our study further suggests that there are other possibly mechanisms by which the litchi peel ethyl acetate extract improves glycaemic control and ameliorates diabetic pathologies, which could be linked to improvement of insulin action and glucose metabolism. In this study, we used fructose and low dose

(40 mg/kg bw) STZ to induce diabetes, resulting in a model characterized by pancreatic β -cell damage and insulin resistance (Wilson and Islam, 2012). Moreover, the association between fructose consumption and insulin resistance in peripheral tissues, including the liver and muscle has been documented (Taskinen et al., 2019). Supporting data from our study showed that diabetes induction significantly ($p < 0.05$) reduced liver and muscle glycogen content (**Table 6.3**). Hepatic and muscle glycogen storage is one of the insulin-mediated mechanisms for glycaemic control and glucose homeostasis (Röder *et al.*, 2016), suggesting that diabetes induction caused impaired insulin action or signaling. However, the litchi peel extract caused a dose dependent increase of liver and muscle glycogen content in the diabetic rats. Although the effect of the litchi peel extract was not significant ($p > 0.05$), the effect of high dose treatment (HDPE) was comparable to that of metformin treatment (**Table 6.3**), suggesting that litchi peel ethyl acetate extract potentiates glycaemic control by promoting glycogen storage.

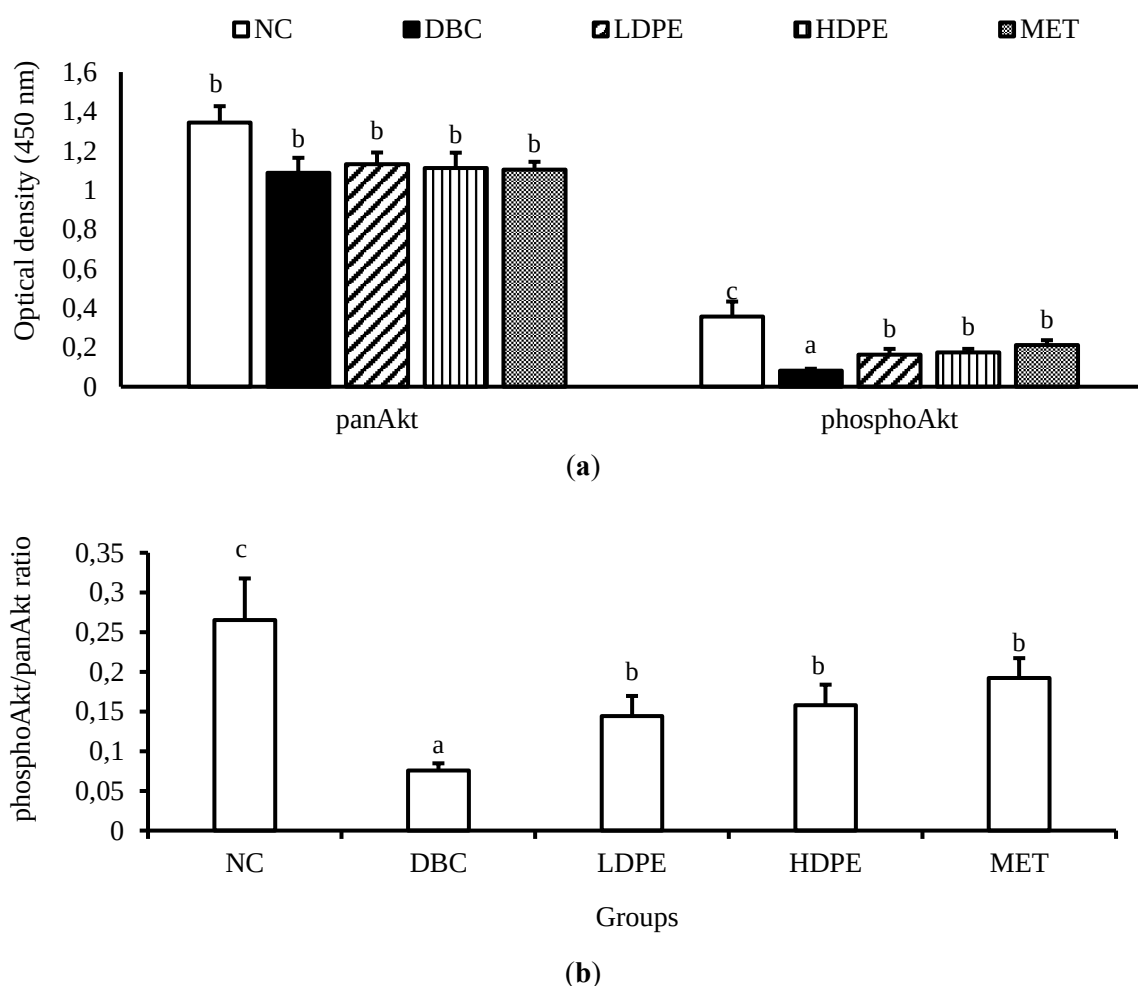


Figure 6.5: Muscle tissue (a) panAkt and phosphoAkt levels and (b) phosphoAkt/panAkt ratio of rats. “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each parameter, statistical comparison was done among the groups. Significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

Furthermore, diabetes induction significantly ($p < 0.05$) reduced muscle hexokinase activity (**Table 6.3**). Hexokinase is a glycolytic enzyme that initiates the cellular utilization of glucose taken up into cells of peripheral tissues (Wasserman, 2022). Glucose uptake and utilization are key insulin-mediated mechanisms for glucose homeostasis and glycaemic control (Röder *et al.*, 2016; Wasserman, 2022), suggesting that diabetes induction suppressed insulin-mediated glucose uptake and utilization in the muscle. Additional molecular investigation showed that diabetes induction significantly ($p < 0.05$) reduced muscle Akt phosphorylation (**Figure 6.5**), a key molecular step in the signaling of cell glucose uptake by insulin (Chang *et al.*, 2004), buttressing the suppressive impact of diabetes induction on insulin-mediated glucose uptake and utilization in the muscle. However, treatment with the peel extract, especially the high dose (HDPE) notably improved muscle hexokinase activity and Akt phosphorylation (**Table 6.3** and **Figure 6.5**). Its modulatory effect on Akt phosphorylation was significant ($p < 0.05$) and comparable to that of metformin treatment, suggesting that the bioactive phytoconstituents in litchi peel ethyl acetate extract exerts glycaemic control effect via modulating cellular insulin signaling, glucose uptake and utilization. Moreover, documented evidence has reported that epicatechin potentiates glycaemic control by modulating the IRS-1/PI3K/Akt signal transduction pathways, thus promoting glucose transporter type 4 (GLUT-4)-mediated cellular glucose uptake (Patanè *et al.*, 2023).

The comparative glycaemic control potential of HDPE relative to metformin may be influenced by the observed multi-mode potential of HDPE. This includes improving pancreatic histology and antioxidant status, insulin secretion and signalling, tissue glycogen production and cellular glucose uptake and utilization. While metformin may be a known standard drug, it may not be able to ameliorate some of the STZ-induced pathologies in the diabetic animals of this study. Documented evidence has shown that metformin could improve hepatic glycogen levels by suppressing glucagon action and gluconeogenesis (Rena *et al.*, 2017). It could also improve insulin signalling, as well as glucose uptake and utilization, and thus improving glycaemic control (Rena *et al.*, 2017). These modes of action are consistent with the glycogen content, Akt phosphorylation and hexokinase activity data of the present study (**Table 6.3** and **Figure 6.5**). However, metformin is not able to affect the STZ-induced pathologies (pancreatic oxidative stress, β -cell damage and reduced insulin production) in the diabetic rats because these are outside its pharmacological mechanisms. This could be the possible reason why metformin could not normalize blood glucose or glycaemic control of the diabetic rat model used in the present study.

6.5 Conclusion

In the present study, the antidiabetic and antioxidant potential of litchi peel ethyl acetate extract was demonstrated in a fructose-STZ model of diabetic rats. The data of the study suggests the extract potentiated glycaemic control by improving pancreatic histology and antioxidant status, improving insulin secretion, promoting glycogen storage and modulating cellular glucose uptake and utilization.

LC-MS analysis further revealed that flavonoids, including catechins, proanthocyanidins and procyanidins were the major phytoconstituents of the extract, which according to documented reports are the possible influencing bioactive components of the extract. The ethyl acetate extract of litchi peel could be a source of bioactive flavonoids that can potentiate glycaemic control in diabetes and mitigate the oxidative-stress related pathologies.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1 General discussion

There are many complications of diabetes and oxidative stress has been reported as a major underlying mediator (Bhatti *et al.*, 2022). While commercial drugs exert glycaemic control by modulating processes that lowers blood glucose, their ability to suppress oxidative stress and mitigate associated diabetic complications may rely a number of other mechanisms that are not directly linked to their pharmacological mode of actions. Medicinal plants, including fruits and vegetables are known to contain dietary antioxidant phytoconstituents, which influences their pharmacological potentials, including mitigating the progression of degenerative diseases, such as diabetes and associated complications (Wallace *et al.*, 2020). Fruits like avocado and litchi are widely cultivated for their commercial value, nutritional value and health benefits. The documented pharmacological potentials (anti-inflammatory, antioxidant, anti-hyperglycaemic, anti-hypertensive, anti-obesogenic, anti-dyslipidaemic, and hepatoprotective) of these fruits suggests their therapeutic relevance in delaying the development and progression of degenerative diseases, which has been linked to some bioactive phenolic and flavonoid compounds (Alkhalaf *et al.*, 2019; Bhuyan, *et al.*, 2019; Zhao *et al.*, 2020). Despite medicinally underutilized, the wastes, including the peel and seeds from these fruits have also been shown to contain these bioactive phenolic and flavonoid compounds, which are key in potentiating the documented anti-inflammatory, antioxidant, anti-hyperglycaemic, anti-hypertensive, anti-obesogenic, anti-dyslipidaemic, and hepatoprotective pharmacological potentials of these wastes (Bhuyan, *et al.*, 2019; Chukwuma *et al.*, 2021; Araujo *et al.*, 2021, Ferreira *et al.*, 2022). Using a bioassay-guided approach, the present study employed computational, *in vitro*, cell-based, and *in vivo* experimental models to elucidate the antioxidant and glycaemic control phytochemistry of the peel of avocado and litchi fruits. Understanding the antioxidant and anti-hyperglycaemic phytochemistry of the peel of these fruits may provide a platform for their effective therapeutic application and thus maximizing the therapeutic potential and utilization of the peel of these fruits.

Preliminary antioxidant assessment of the peel crude extracts showed that the avocado methanol extract and the litchi ethyl acetate extract had the highest phenolic and flavonoid content and the most potent radical scavenging and anti-lipid (linoleic acid) peroxidative activity compared to the other corresponding solvent extracts from both peel extracts (**Tables 4.1 and 5.1**). Plant polyphenols, on the other hand, are known radical scavengers, due to their ability to transfer hydroxyl H-atom and form stable phenoxy radicals (Chen *et al.*, 2020), suggesting the correlation between the phenolic content and antioxidant capacity of the avocado methanol extract and the litchi ethyl acetate extract.

documented evidence has also shown correlations between the phenolic contents of avocado peel extract and its radical scavenging and in vitro antioxidant capacity (Antasionasti *et al.*, 2017; Lyu *et al.*, 2023).

Furthermore, the litchi peel ethyl acetate extract had the most potent ($p < 0.05$) α -glucosidase inhibitory activities relative to the other litchi peel extracts, although the methanol extract of litchi peel also showed appreciable α -glucosidase (**Table 5.1**). The enzyme inhibitory effect of the polar extracts of litchi peel has been linked to the presence of notable flavonoids such as quercetin glycosides, proanthocyanidin, catechins and derivatives, etc. (Tanaka *et al.*, 2024), which are also known to exert enzyme inhibitory action (Dirir *et al.*, 2022). While the isolation of bioactive flavonoids (epicatechin and proanthocyanidins) from litchi peel, have mostly been done on the hydroalcoholic extracts (Zhao *et al.*, 2006; Zhao *et al.*, 2007; Rong *et al.*, 2017; Queiroz *et al.*, 2018; Chukwuma *et al.*, 2021; Tanaka *et al.*, 2024; Yang *et al.*, 2024), the present study demonstrates that the ethyl acetate extract of litchi peel could also be an underexplored source of bioactive flavonoids (epicatechin and proanthocyanidins) with enzyme inhibitory action. α -Glucosidase inhibitors exert glycaemic control by reducing postprandial blood glucose (Kashtoh *et al.*, 2022), suggesting the glycaemic potential of litchi peel ethyl acetate extract

Subjecting the potent extract (methanol extract) of avocado fruit peel resulted in 9 fractions (fraction 2 to 10). Antioxidant assessment of the fractions from avocado peel methanol extract showed that fraction 8 (eluted with hexane:chloroform:methanol volume ratio of 3:6.5:0.5, respectively) consistently had the highest ($p < 0.05$) total phenol content, as well as DPPH and ABTS radical scavenging and Fe^{3+} reducing antioxidant activity (**Table 4.2**). The antioxidant activity of fraction 8 was statistically comparable to that of ascorbic acid and/or Trolox (**Table 4.2**), suggesting this fraction contains antioxidant constituents. Consistent with previous phytochemical data of avocado peel (Hirasawa *et al.*, 2008; Rodríguez-Martínez *et al.*, 2021), LC-MS analysis of fraction 8 showed the predominant presence of chlorogenic acid (5-CQA) and 1-*O*-caffeoylquinic acid (1-CQA) (**Table 4.3** and **Figure 4.1**). In fact the radical scavenging activity of chlorogenic acid was comparable to that of fraction 8 (**Table 4.4** and **Figure 4.2a**), suggesting that chlorogenic acid may strongly influence the antioxidant effect of fraction 8. Moreover, chlorogenic acid is a known dietary phenolic acid with antioxidant and anti-inflammatory potentials (Liang *et al.*, 2015; Wang *et al.*, 2022; Huang *et al.*, 2023). Further computational DFT analysis suggests that the favoured radical scavenging mechanism of chlorogenic acid is a single electron transfer followed by proton transfer at 3'OH and 4'OH positions (**Table 4.5** and **Figure 4.6**).

Further cellular antioxidant assessment show that both chlorogenic acid and fraction 8 suppressed lipid peroxidation (**Table 4.4** and **Figures 4.2b** and **4.3a**) and modulated the activity of catalase and SOD activity (**Figure 4.3b**) in oxidatively stressed cells, which may be linked to the ability of chlorogenic acid to mop radical by-products of lipid peroxidation and modulate antioxidant status (Zhou *et al.*, 1993;

Petrovic *et al.*, 2020; Wang *et al.*, 2020). In LPS-treated RAW 264.7 macrophages, it appears that chlorogenic acid influenced the fraction's suppressive effect on ROS (**Table 4.4** and **Figure 4.4**) and NO (**Table 4.4** and **Figure 4.5a**) production, reiterating the ROS mopping capacity of avocado peel. Moreover, the modulatory action of chlorogenic acid on biological redox status has been documented (Zhou *et al.*, 1993; Liang *et al.*, 2015; Wang *et al.*, 2020; Wang *et al.*, 2022). The suppressive effect of fraction 8 and chlorogenic acid on NO production may be linked to an anti-inflammatory potential. This because immune myeloid cells, like macrophages, are known to produce more cytokines and NO in response to inflammatory signals (Palmieri *et al.*, 2020). Moreover, a similar suppressive effect by avocado peel hydroalcoholic extract have been reported (Tremocoldi *et al.*, 2018) and chlorogenic acid may be a key influencer, considering its documented suppressive effect on the production and action of inflammatory mediators, including TNF- α , IL-1 β , IL-6, IL-8, NO, and PGE2 (Huang *et al.*, 2023).

Subjecting the potent extract (ethyl acetate extract) of litchi fruit peel, on the other hand, resulted in 8 fractions (fraction 1 to 8). Qualitative phytochemical and glycaemic control assessments showed that the α -glucosidase inhibitory potency of the fractions correlated with their flavonoid contents, with fraction 5 (obtained from DCM:MeOH:water; 7:3:0.281 v/v/v solvent system) outperforming other fractions (**Table 5.2**). LC-MS profiling of the fraction showed the notable presence of epicatechin, among other flavonoids (**Table 5.3** and **Figure 5.1**), which reflects the predominant flavonoids (biflavonoids and polyflavonoids such as epicatechin, cinnamtannin B2, procyanidin B5 and proanthocyanidin A2) observed in the parent ethyl acetate extract (**Table 6.1** and **Figure 6.1**) and the flavonoids that have been documented in litchi peel (Wu *et al.*, 2019; Xu *et al.*, 2019; Şöhretoğlu *et al.*, 2023). The predominant presence of epicatechin in fraction 5 may have influenced its more robust ($p < 0.05$) α -glucosidase inhibitory activity relative to the other fractions (**Table 5.2**). Moreover, the enzyme inhibitory activity of epicatechin and derivatives has been documented (Wu *et al.*, 2019; Xu *et al.*, 2019; Şöhretoğlu *et al.*, 2023).

Subjecting fraction 5 to Sephadex chromatography yielded 7 sub-fractions, which were assessed for their radical scavenging, anti-lipid peroxidative, α -glucosidase inhibitory, and cellular glucose uptake activities. The anti-lipid peroxidative and α -glucosidase inhibitory activities of sub-fractions 4 and 5 significantly ($p < 0.05$) outperformed the activities of the other sub-fractions (**Table 5.4**). In fact, at a tested concentration of 20 $\mu\text{g/mL}$, their anti-lipid peroxidative and α -glucosidase activities were comparable to that of Trolox and acarbose, respectively. The data suggests that the epicatechin principle in fraction 5 (**Table 5.3** and **Figure 5.1**) may have been eluted in sub-fractions 4 and 5, which influenced their potent activities. Moreover, the protective effect of epicatechin and derivatives on lipid peroxidative damage (Terao *et al.*, 1994; Shaki *et al.*, 2017), as well as their α -glucosidase inhibitory effects (Wu *et al.*, 2019; Xu *et al.*, 2019; Şöhretoğlu *et al.*, 2023) have been documented.

Furthermore, sub-fractions 4 and 5 showed stronger ($p < 0.05$) glucose uptake modulatory activities, relative to the other sub-fractions in Chang cells (**Table 5.4**). Although, sub-fraction 4 was not as potent as sub-fraction 5 ($p < 0.05$). Cellular glucose uptake is a key mechanism in physiological glucose homeostasis, which is impaired during T2D due to insulin resistance (Galicia-Garcia *et al.*, 2020). Thus, the potent glucose uptake modulatory activity of sub-fraction 4 suggests it contains flavonoid(s) that can potentiate glycaemic control via improving insulin signalling and cellular glucose uptake and utilization. Epicatechin was the most plausible influencing bioactive flavonoids of sub-fraction 4, since it was the most abundant flavonoid in the parent fraction (fraction 5) (**Table 5.3** and **Figure 5.1**). It is a dietary supplement reported to improve cellular insulin sensitivity and glucose tolerance in obese and T2D individuals (Cremonini *et al.*, 2019). Hence, sub-fraction 4 was probed further to isolate bioactive epicatechin.

Bioactivity assessment of the isolated epicatechin showed that it demonstrated a dose-dependent inhibitory action ($IC_{50} = 18.9 \mu M$) on LPS-induced ROS production in RAW 264.7 macrophages, which was statistically comparable ($p > 0.05$) to that of ascorbic acid ($IC_{50} = 9.57 \mu M$) (**Table 5.6** and **Figure 5.3**), reiterating the antioxidant potency of epicatechin. LPS-induced ROS production has been linked to pro-inflammatory signalling, which can disrupt cellular processes and cause oxidative tissue injuries due to excessive production of superoxide ions and hydrogen peroxide (Hsu *et al.*, 2002). Hence, it is plausible that the inhibitory effect of epicatechin on LPS-induced ROS production in RAW 264.7 macrophages was linked to both antioxidant and anti-inflammatory capacities. Moreover, the inhibitory effect of epicatechin on LPS-induced production of proinflammatory mediators and cytokines in RAW 264.7 macrophages has been reported (Wang *et al.*, 2014).

Furthermore, the isolated epicatechin demonstrated a dose-dependent glycaemic control potential. It had an inhibitory effect ($IC_{50} = 18.9 \mu M$) on α -glucosidase (**Table 5.6** and **Figure 5.4**), suggesting it could be a key flavonoid influencing the α -glucosidase inhibitory activity of the potent sub-fraction(s)/fraction(s) and extract of litchi fruit peel. In Chang cells, epicatechin exerted dose-dependent modulatory effect ($EC_{50} = 78.5 \mu M$) on glucose uptake (**Table 5.6** and **Figure 5.5**), suggesting epicatechin may be effective in promoting cellular glucose uptake in cells of peripheral tissues, and may have influenced the glucose uptake activity of the potent sub-fractions 4 and 5 (**Table 5.3**). Moreover, documented evidence suggests that epicatechin may enhance glucose uptake by modulating insulin signalling via the PI3K/AKT signalling pathway and modulating the translocation of glucose transporters (Xu *et al.*, 2019; Chun *et al.*, 2022). Further studies are, however, needed to investigate this speculation.

In vivo, the litchi peel ethyl acetate ameliorated fructose/STZ-induced diabetic pathologies, which may be influenced by the rich flavonoid profile of the extract. Although not normalized, the high dose of the litchi peel ethyl acetate extract (HDPE) significantly ($p < 0.05$) reduced diabetic polyphagia (**Table**

6.2), suggesting a notable ameliorative effect of the litchi peel ethyl acetate extract, which can be further investigated long-term. Weekly NFBG data showed that the peel extract dose dependently reduced NFBG over the treatment period (**Figure 6.2**). Notably, the HDPE caused significant ($p < 0.05$) reduction in the 2nd and 4th week of the intervention period, which was statistically comparable to that of metformin treatment, suggesting the blood glucose lowering potential of the peel's ethyl acetate extract. Furthermore, the extract also exhibited a dose dependent glucose tolerance effect (**Figure 6.3a**), with the effect of the HDPE being significant ($p < 0.05$) relative to the untreated diabetic animals (**Figure 6.3b**).

While the blood glucose lowering effect of litchi peel ethyl acetate extract has been previously documented, the effect was demonstrated in alloxan-induced diabetic rats (Chauhan *et al.*, 2021). The alloxan-induced and STZ-induced diabetic models are characterized by oxidative stress-mediated pancreatic β -cell damage and impaired insulin secretion (Lenzen, 2008), which suggests that the previously documented blood glucose lowering effect of litchi peel ethyl acetate extract (Chauhan *et al.*, 2021) may be influenced by an improvement of pancreatic β -cell damage, among other possible mechanisms. This is because supporting antioxidant data from the study by Chauhan *et al.* (2021) showed that the ethyl acetate extract improved the pancreatic antioxidant status of the alloxan-induced diabetic rats. Histology data comparing the pancreas of the normal rats (NC) with that of the untreated (DBC) and treated (LDPE, HDPE and MET) diabetic rats further reiterate the possible ameliorative effect of litchi peel ethyl acetate extract on chemical (STZ)-induced pancreatic damage (**Figure 6.4**). This ameliorative effect further reflects the ability of the HDPE to significantly ($p < 0.05$) improve insulin secretion in diabetic rats as shown by the significantly ($p < 0.05$) higher serum insulin level compared to the untreated diabetic rats (**Table 6.3**).

It is plausible that the ameliorative effect of the HDPE on STZ-induced pancreatic damage may be linked to their pancreatic antioxidant capacities. The data of this study showed that the HDPE treatment significantly ($p < 0.05$) reduced pancreatic lipid peroxidation and concomitantly increased ($p < 0.05$) systemic and pancreatic catalase and SOD activities (**Table 4**). Catalase and SOD are antioxidant enzymes that respectively catalyze the reduction deleterious superoxide ion and hydrogen peroxide (Lee and Park, 2021). Hence, the significantly higher pancreatic catalase and SOD activity of rats in the HDPE group, relative to the untreated diabetic rats suggests that the HDPE ameliorated STZ-induced ROS damage in the pancreatic tissue by mopping deleterious ROS. The potent in vivo antioxidant activity of the HDPE may be linked to notable presence of bioactive flavonoids including catechins, proanthocyanidins and procyanidins (**Table 6.1 and Figure 6.1**). Moreover, the hydroxyl radical and superoxide anion scavenging activity of proanthocyanidin B4, proanthocyanidin B2 and epicatechin isolated from litchi peel has been reported (Zhao *et al.*, 2006), while the modulatory effect of catechins and proanthocyanidins in vivo antioxidant status and antioxidant enzymes activity have been documented (Patanè *et al.*, 2023). Furthermore, the key bioactive flavonoids present in the peel extracts

may also influence its modulatory effect on insulin secretion. This is because a study has shown that epicatechin, a major flavonoid in the peel extract (**Table 6.1 and Figure 6.1**), modulates glucose-stimulated insulin secretion in INS-1 cells (Yang and Chan, 2018).

Other than the improvement of pancreatic antioxidant capacity, histology and insulin secretion, our study further suggests that there are other possibly mechanisms by which the litchi peel ethyl acetate extract improves glycaemic control and ameliorates diabetic pathologies, which could be linked to improvement of insulin action and glucose metabolism. In this study, we used fructose and low dose (40 mg/kg bw) STZ to induce diabetes, resulting in a model characterized by pancreatic β -cell damage and insulin resistance (Wilson and Islam, 2012). Moreover, the association between fructose consumption and insulin resistance in peripheral tissues, including the liver and muscle has been documented (Taskinen *et al.*, 2019). Supporting data from our study showed that diabetes induction significantly ($p < 0.05$) reduced liver and muscle glycogen content (**Table 6.3**). Hepatic and muscle glycogen storage is one of the insulin-mediated mechanisms for glycaemic control and glucose homeostasis (Röder *et al.*, 2016), suggesting that diabetes induction caused impaired insulin action or signaling. However, the litchi peel extract caused a dose dependent increase of liver and muscle glycogen content in the diabetic rats. Although the effect of the litchi peel extract was not significant ($p > 0.05$), the effect of high dose treatment (HDPE) was comparable to that of metformin treatment (**Table 6.3**), suggesting that litchi peel ethyl acetate extract potentiates glycaemic control by promoting glycogen storage.

Furthermore, treatment with the peel extract, especially the high dose (HDPE) notably improved muscle hexokinase activity and Akt phosphorylation (**Table 6.3 and Figure 6.5**). Its modulatory effect on Akt phosphorylation was significant ($p < 0.05$) and comparable to that of metformin treatment. Hexokinase is a glycolytic enzyme that initiates the cellular utilization of glucose taken up into cells of peripheral tissues (Wasserman, 2022). Glucose uptake and utilization are key insulin-mediated mechanisms for glucose homeostasis and glycaemic control. Also, Akt phosphorylation is a key molecular step in the signaling of cell glucose uptake by insulin (Chang *et al.*, 2004). Hence the modulatory effect of the litchi peel ethyl acetate extract suggest that its bioactive constituents exert glycaemic control effect via modulating cellular insulin signaling, glucose uptake and utilization. Moreover, documented evidence has reported that epicatechin potentiates glycaemic control by modulating the IRS-1/PI3K/Akt signal transduction pathways, thus promoting glucose transporter type 4 (GLUT-4)-mediated cellular glucose uptake (Patanè *et al.*, 2023).

7.2 General conclusion

In the present study, a bioassay-guided approach was used to elucidate the antioxidant and glycaemic control phytochemistry of the peel of avocado and litchi fruits. For avocado fruit peel, methanol solvent

was effective in extracting antioxidant phenolics. Fractionation of the peel's methanol extract yielded a fraction that had a promising ability to scavenge free radical, inhibit lipid peroxidation, suppress ROS and NO production and modulate antioxidant enzyme activity. The fraction also showed the predominant presence of chlorogenic acid. Data further showed that chlorogenic acid had antioxidant activities that were comparable to that of the fraction and ascorbic acid. It scavenged DPPH radical through a single electron transfer, followed by proton transfer at its carbons 3'OH and 4'OH positions.

For litchi fruit peel, ethyl acetate solvent effectively extracted flavonoids, such as including catechins, proanthocyanidins and procyanidins, which potentiated appreciable antioxidant and carbohydrate digestive enzymes inhibitory activities. Epicatechin isolated from the peel's ethyl acetate extract demonstrated a dose-dependent inhibitory effect on α -glucosidase, modulatory effect on cellular glucose uptake and inhibitory effect on cellular ROS production, suggesting it is a potent antioxidant and glycaemic control constituent of litchi peel. *In vivo*, peel's ethyl acetate extract exerted antioxidant and glycaemic control effects by improving pancreatic histology and antioxidant status, improving insulin secretion, promoting glycogen storage and modulating cellular glucose uptake and utilization, suggesting the ethyl acetate extract of litchi peel could be a source of bioactive flavonoids that can potentiate glycaemic control in diabetes and mitigate the oxidative-stress related pathologies.

In conclusion, considering this study is bioassay-guided, it is logical to conclude that chlorogenic acid strongly influences the antioxidant capacity of avocado fruit peel, while flavonoids including catechins, proanthocyanidins and procyanidins are some of the predominant constituents influencing the glycaemic control and antioxidant properties of litchi peel. Future studies elucidating the medicinal phytochemistry of the wastes from other fruits are, therefore recommended. Understanding the medicinal phytochemistry of the fruit wastes may provide a platform for their effective therapeutic application and thus maximizing the therapeutic potential and utilization of the under-utilized wastes from fruits.

REFERENCES

- Abdullahi, M., Musa, M., Abdullahi, I., Sani, M., Atiku, I. Catechin from the leaf extract of *Ziziphus mucronata* Willd. (Rhamnaceae). *Niger. J. Pharm.Sci.* 2017, 16(2), 01– 05.
- Adelusi, T., Oboh, G., Akinyemi, A., Ajani, R., Bakare, O. Avocado Pear Fruits and Leaves Aqueous Extracts Inhibit A-Amylase, A-Glucosidase and Snp Induced Lipid Peroxidation – An Insight into Mechanisms Involve in Management of Type 2 Diabetes. *Int. J. Nat. Sci.* 2014; 3. 21 - 34.
- Afam, J., Silungwe, H., Takalani, T., Omolola, O., Udeh, O., Anyasi, A. Antioxidant-rich natural fruit and vegetable products and human health. *Int. J. Food Prop.* 2021, 24, 41–67.
- Aguirre, V., Uchida, T., Yenush, L., Davis, R., White, F. The c-Jun NH (2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser (307) *J. Biol. Chem.* 2000; 275(12):9047–9054. doi: 10.1074/jbc.275.12.9047.
- Akan, S. Phytochemicals in avocado peel and their potential uses. *Food and Health*, 2021; 7(2), 138-149. <https://doi.org/10.3153/FH21015>
- Akmal, M. Patel, P., Wadhwa, R. Alpha Glucosidase Inhibitors. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024; Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK557848/>
- Akshaya, M., Sekar, S., Nithyalakshmi and Meenakshi, N. Effect of pre-treatments on the phytochemical composition of watermelon (*Citrullus lanatus*) rind. *Int. Food Res. J.* 2018, 25(5), 1920-1924.
- Alicic, R., Rooney, M., Tuttle, K. Diabetic kidney disease. *Clin J Am Soc Nephrol* 2017; 12: 2032–2045.
- Alrasheid, A., Mohamed, A., Mohieldin, E., Eldein, K., Yassin, L., Widdatallh, M., Alnour, M., Eltilib, S., Ahmed, S., & Ayoub, S. Phytochemical investigation and assessment of antimicrobial, anti-inflammatory and antioxidant activities of Sudanese *Citrus paradisi* peel extract. *J. Pharmacognosy Phytother.* 2019; 11(1), 1-8
- Amado, D., Helmann, G., Detoni, A., Carvalho, S., Aguiar, C., Martin, C., Tiuman, T., Cottica, S. Antioxidant and antibacterial activity and preliminary toxicity analysis of four varieties of avocado (*Persea americana* Mill.). *Braz. J. Food Technol.* 2019, 22, 1-11
- American Diabetes Association Position Statement: Standards of Medical Care in Diabetes—2015. *Diabetes Care* 2015;38(Suppl. 1): S1–S94.
- American Diabetes Association. Approaches to glycemic treatment. Standards of medical care in diabetes - 2016. *Diabetes Care* 2016; 39(Supplement 1): S52-S59.

- American Diabetes Association Professional Practice Committee. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2022. *Diabetes Care*, 2022, 45(Suppl 1), S17–S38. <https://doi.org/10.2337/dc22-S002>.
- American Diabetes Association. Microvascular complications and foot care: Standards of medical care in diabetes—2018. *Diabetes Care* 2018;41: S105-18.
- American Diabetes Association. Older adults: Standards of Medical Care in Diabetes-2019. *Diabetes Care* 2019, 42, S139–S147.
- American Diabetes Association. Peripheral arterial disease in people with diabetes. *Diabetes Care* 2003; 26: 3333-3341
- Ang, Y., Sia, W., Khoo, H., Yim, H. Antioxidant potential of Carica papaya peel and seed. *Focusing on Modern Food Industry*. 2012;1(1):11–16
- Angelidi, A., Filippaios, A., Mantzoros, C. Severe insulin resistance syndromes. *J Clin Invest*. 2021 Feb 15;131(4):e142245. doi: 10.1172/JCI142245.
- Antasionasti, I., Riyanto, S., Rohman, A. Antioxidant activities and phenolics contents of avocado (*Persea americana* Mill.) peel *in vitro*. *J. Med. Plant Res*. 2017, 11, 55–61.
- Antonetti, D., Klein, R., Gardner, T. Diabetic retinopathy. *N Engl J Med*. 2012;366(13):1227-39
- Aqilah, N., Rovina, K., Felicia, W., Vonnice, A Review on the Potential Bioactive Components in Fruits and Vegetable Wastes as Value-Added Products in the Food Industry. *Molecules*. 2023; Mar 14;28(6):2631.
- Araujo, R., Rodríguez-Jasso, R., Ruíz, H., Govea-Salas, M., Pintado, M., Aguilar, C. Recovery of bioactive components from avocado peels using microwave-assisted extraction. *Food Bioprod. Process*. 2021, 127, 152–161.
- Archuleta T., Lemieux, A., Saengsirisuwan, V., Teachey, M., Lindborg, K., Kim, J., Henriksen, E. Oxidant stress-induced loss of IRS-1 and IRS-2 proteins in rat skeletal muscle: role of p38 MAPK, *Free Radic Biol Med* 47(10) 2009; 1486-93.
- Awuchi, G. “Medicinal Plants, Bioactive Compounds, and Dietary Therapies for Treating Type 1 and Type 2 Diabetes Mellitus.” *Pharmacognosy - Medicinal Plants*. 2021: n. pag.
- Azarova, E., Alexey, P., and Yu. K. “Molecular Genetics of Abnormal Redox Homeostasis in Type 2 Diabetes Mellitus.” *Int. J. Mol. Sci*. 2023; n. pag.
- Azizan, A., Xin, L., Abdul A., Maulidiani M., Mediani A., Abdul Ghafar S., Zulaikha Zolkeflee N., Abas F. Potentially Bioactive Metabolites from Pineapple Waste Extracts and Their Antioxidant

- and α -Glucosidase Inhibitory Activities by ¹H NMR. *Foods*. 2020; Feb 11;9(2):173. doi: 10.3390/foods9020173.
- Azzam, C., L., Lugo-Gavidia, J., Nolde, V., Matthews, M. Schlaich, Bexagliflozin for type 2 diabetes: an overview of the data, *Expet Opin. Pharmacother*. 22 2021; 2095–2103.
- Bancks, M., Akhabue, E., Rana, J., Reis, J., Schreiner, P., Yano, Y., Lewis, C. Sex differences in cardiovascular risk factors before and after the development of type 2 diabetes and risk for incident cardiovascular disease. *Diabetes Res Clin Pract*. 2020, 166:108334. 10.1016/j.diabres.2020.108334
- Banday, M., Sameer, A., Nissar, S. Pathophysiology of diabetes: An overview. *Avicenna J Med*. 2020 Oct 13;10(4):174-188. doi: 10.4103/ajm.ajm_53_20.
- Baniwal, P., Mehra, R., Kumar, N., Sharma, S., Kumar, S. Cereals: Functional constituents and its health benefits. *Pharma Innovation* 2021;10(3):01-7.
- Bardaweel, K., Gul, M., Alzweiri, M., Ishaqat, A., ALSalamat, A., Bashatwah, M. Reactive oxygen species: The dual role in physiological and pathological conditions of the human body. *Eurasian J. Med*. 2018, 50, 193–201.
- Barros, H., Baseggio, A., Angolini, C., Pastore, G., Cazarin, C., Marostica-Junior, M. Influence of different types of acids and pH in the recovery of bioactive compounds in Jabuticaba peel (*Plinia cauliflora*). *Int. Food Res. J*. 2019, 124, 16–26.
- Basu, S., Yoffe, P., Hills, N., Lustig, R. The relationship of sugar to population-level diabetes prevalence: an econometric analysis of repeated cross-sectional data. *PLoS One*. 2013;8(2):e57873. doi: 10.1371/journal.pone.0057873.
- Beadon, K., Guimarães-Costa, R., Léger, M. Multifocal motor neuropathy. *Curr Opin Neurol*. 2018 Oct;31(5):559-564. doi: 10.1097/WCO.0000000000000605.
- Beckman, J., Creager, M. Vascular complications of diabetes. *Circ Res* 2016; 118: 1771–1785.
- Behl, T., Bungau, S., Kumar, K., Zengin, G., Khan, F., Kumar, A., Kaur, R., Venkatachalam, T., Tit, M., Vesa, M., Barsan, G., Mosteanu, E. Pleotropic Effects of Polyphenols in Cardiovascular System. *Biomed Pharmacother*. 2020 Oct;130:110714. doi: 10.1016/j.biopha.2020.110714.
- Belančić, A., Klobučar, S., Rahelić, D. Current Obstacles (With Solutions) in Type 2 Diabetes Management, Alongside Future Directions. *Diabetology* 2023, 4, 376-378. <https://doi.org/10.3390/diabetology4030031>

- Bergheanu, S., Bodde, M., Jukema, J. Pathophysiology and treatment of atherosclerosis: current view and future perspective on lipoprotein modification treatment. *Neth Heart J.* 2017; 25:231–242. doi: 10.1007/s12471-017-0959-2
- Bertling, I., Tesfay, S., Bower, J. Antioxidants in ‘Hass’ avocado. *South Afr. Avocado Grow. Assoc. Yearb.* 2007, 30, 17–19
- Bhatti, S., Sehrawat, A., Mishra, J., Sidhu, S., Navik, U., Khullar, N., Kumar, S., Bhatti, K., Reddy, H. Oxidative stress in the pathophysiology of type 2 diabetes and related complications: Current therapeutics strategies and future perspectives. *Free Radic. Biol. Med.* 2022, 184, 114–134. <https://doi.org/10.1016/j.freeradbiomed.2022.03.019>
- Bhuyan, J., Alsherbiny, A., Perera, S., Low, M., Basu, A., Devi, A., Barooah, S., Li, G., Papoutsis K. The Odyssey of Bioactive Compounds in Avocado (*Persea americana*) and Their Health Benefits. *Antioxidants.* 2019; 8(10):426. <https://doi.org/10.3390/antiox8100426>
- Birben, E., Sahiner, M., Sackesen, C., Erzurum, S., Kalayci, O. Oxidative stress and antioxidant defense. *World Allergy Organ J.* 2012, 5, 9–19.
- Biswas, T., Behera, B., Madhu, N. Technology in the Management of Type 1 and Type 2 Diabetes Mellitus: Recent Status and Future Prospects. In: Noor, R. (eds) *Advances in Diabetes Research and Management.* Springer, 2023; Singapore. https://doi.org/10.1007/978-981-19-0027-3_6
- Black, C., Donnelly, P., McIntyre, L., Royle, P., Shepherd, J., Thomas, S. Meglitinide analogues for type2 diabetes mellitus. *Cochrane Database Syst. Rev.* 2007; 2, 1–2.
- Bösenberg, L., & van Zyl, D. The mechanism of action of oral antidiabetic drugs: A review of recent literature. *Endocrinol Diabetes Metab J South Africa,* 2008; 13(3), 80–88. <https://doi.org/10.1080/22201009.2008.10872177>
- Bournat, C., Brown, W. Mitochondrial dysfunction in obesity. *Curr Opin Endocrinol Diabetes Obes.* 2010;17(5):446–52.
- Bower, R., Hay, D. Amylin structure-function relationships and receptor pharmacology: implications for amylin mimetic drug development. *Br J Pharmacol.* 2016; Jun;173(12):1883-98. doi: 10.1111/bph.13496.
- Boyadzhieva, S., Georgieva, S., Angelov, G. Optimization of the extraction of natural antioxidants from avocado seeds. *Bulg. Chem. Commun.* 2018; 50, 80–84.
- Brglez, M., Knez Hrnčič, M. Škerget, Ž. Knez and U. Bren, Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects, *Molecules*, 2016, 21, 901.

- Brown, J., de Banate, A., Rother, I. Artificial sweeteners: a systematic review of metabolic effects in youth. *Int J Pediatr Obes*. 2010 Aug;5(4):305-12. doi: 10.3109/17477160903497027.
- Brown, J., Walter, M., Rother, I. Ingestion of diet soda before a glucose load augments glucagon-like peptide-1. *Diab care* 2009; 32(12):2184 - 2186.
- Brownrigg, J., Apelqvist, J., Bakker, K., Schaper, N., Hinchliffe, R. Evidence-based Management of PAD & the Diabetic Foot, *Eur J Vasc Endovasc Surg*, Volume 45, Issue 6, 2013, Pages 673-681, ISSN 1078-5884, <https://doi.org/10.1016/j.ejvs.2013.02.014>.
- Butt, M. "Management and Treatment of Type 2 Diabetes." *Intl. J. of Computations, Information and Manufacturing* 2022; n. pag.
- Calderón-Oliver, M., Escalona-Buendía, B., Medina-Campos, N., Pedraza-Chaverri, J., Pedroza-Islas, R., Ponce-Alquicira, E. Optimization of the antioxidant and antimicrobial response of the combined effect of nisin and avocado byproducts. *Lwt-Food Sci. Technol*. 2016, 65, 46–52
- Camila, K., Rúbia, C., Diego de Souza, L., Anacharis, de Sá-Nakanishi, Geferson de Almeida Gonçalves, Flavio, S., Charles, H., Lillian, B., Isabel, C., Ferreira, B., Rosane, P., Potential anti-diabetic properties of Merlot grape pomace extract: An *in vitro*, *in silico* and *in vivo* study of α -amylase and α -glucosidase inhibition, *Int. Food Res*, Volume 137, 2020, 109462, ISSN 0963-9969, <https://doi.org/10.1016/j.foodres.2020.109462>
- Cano-Gómez, I., Alonso-Castro, J., Carranza-Alvarez, C., Wong-Paz, E. Advancements in *Litchi chinensis* Peel Processing: A Scientific Review of Drying, Extraction, and Isolation of Its Bioactive Compounds. *Foods*. 2024, 13(10), 1461. <https://doi.org/10.3390/foods13101461>
- Carvajal-Zarrabal, O.; Nolasco-Hipolito, C.; Aguilar-Uscanga, M.G.; Melo-Santiesteban, G.; Hayward-Jones, P.M.; Barradas-Dermitz, D.M. Avocado oil supplementation modifies cardiovascular risk profile markers in a rat model of sucrose-induced metabolic changes. *Dis. Markers* 2014, 386425.
- Castro-Vazquez, L., Alañón, E., Rodríguez-Robledo, V., Pérez-Coello, S., Hermosín-Gutierrez, I., Díaz-Maroto, C., Jordán, J., Galindo, F., Arroyo-Jiménez M. Bioactive Flavonoids, Antioxidant Behaviour, and Cytoprotective Effects of Dried Grapefruit Peels (*Citrus paradisi Macf.*). *Oxid Med Cell Longev*. 2016; 8915729. doi: 10.1155/2016/8915729.
- Cavati, Guido, Filippo Pirrotta, Daniela Merlotti, Elena Ceccarelli, Marco Calabrese, Luigi Gennari and Christian Mingiano. "Role of Advanced Glycation End-Products and Oxidative Stress in Type-2-Diabetes-Induced Bone Fragility and Implications on Fracture Risk Stratification." *Antioxidants* 12 2023; n. pag

- Cem, S., Volkan, Y. Treat Obesity to Treat Type 2 Diabetes Mellitus. *Diabetes Ther.* 2024, <https://doi.org/10.1007/s13300-024-01536-3>
- Centers for Disease Control and Prevention. National Diabetes Statistics Report; U.S. Dept of Health and Human Services: Atlanta, GA, USA 2020. Available online: <https://cdc.gov/diabetes/data/statistics-report/index.html>
- Cerf, E. Beta cell dysfunction and insulin resistance. *Front. Endocrinol. (Lausanne)* 2013; 4, 37.
- Chang, L., Chiang, H., & Saltiel, R. Insulin Signaling and the Regulation of Glucose Transport. *Mol Med* 2004 10, 65–71. <https://doi.org/10.2119/2005-00029.Saltiel>
- Chaudhury, A., Duvoor, C., Reddy, S., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, S., Montales, T., Kuriakose, K., Sasapu, A., Beebe, A., Patil, N., Musham, K., Lohani, P., Mirza, W. Clinical Review of Antidiabetic Drugs: Implications for Type 2 Diabetes Mellitus Management. *Front. Endocrinol.* 2017, 8, 6
- Chauhan, S., Gupta, S., Yasmin, S., Saini, M. Antihyperglycemic and Antioxidant Potential of Plant Extract of *Litchi chinensis* and *Glycine max*. *Int. J. Nutr. Pharmacol. Neurol. Dis.* 2021, 11(3), 225–233, https://doi.org/10.4103/ijnpnd.ijnpnd_13_21
- Chauhan, S., Kaur, N., Kishore, L., Singh, R. Pharmacological evaluation of the anti-inflammatory and analgesic potential of *Litchi chinensis Gaertn.(Sonn.)* *Int J Pharmacy Pharm Sci* 2014; 6:116-9.12.
- Chen, J., Yang, J., Ma, L., Li, J., Shahzad, N., Kim, K. Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Sci. Rep.* 2020, 10(1), 2611. <https://doi.org/10.1038/s41598-020-59451-z>
- Chen, L. G., Chang, C. W., Tsay, J. G., & Weng, B. B. Hepatoprotective effects of litchi (*Litchi chinensis*) procyanidin A2 on carbon tetrachloride-induced liver injury in ICR mice. *Exp Ther Med*, 2017 13(6), 2839–2847. <https://doi.org/10.3892/etm.2017.4358>
- Chen, Y., Li, H., Zhang, S., Yang, C., Mai, Z., Hu, X., Gao, Z., & Deng, H. Anti-myocardial ischemia effect and components of Litchi pericarp extracts. *Phytother. Res.*, 2017 31(9), 1384–1391. <https://doi.org/10.1002/ptr.5865>
- Choi, S., Lee, J., Kyung, M., Youn, J., Oh, J., & Whang, W. Anti-diabetic functional food with wasted litchi seed and standard of quality control. *Appl. Biol. Chem* 2017; 60, 197–204. <https://doi.org/10.1007/s13765-017-0269-9>

- Chougale, A., Ghadyale, V., Panaskar, S., & Arvindekar, A. Alpha glucosidase inhibition by stem extract of *Tinospora cordifolia*. *J. Enzyme Inhib. Med. Chem.*, 2009; 24(4), 998–1001. <https://doi.org/10.1080/14756360802565346>
- Chukwuma, C.I. Antioxidative, Metabolic and Vascular Medicinal Potentials of Natural Products in the Non-Edible Wastes of Fruits Belonging to the *Citrus* and *Prunus* Genera: A Review. *Plants*. 2024; 13(2):191. <https://doi.org/10.3390/plants13020191>
- Chukwuma, I., Izu, O., Chukwuma, S., Samson, S., Makhafola, J., & Erukainure, L. A review on the medicinal potential, toxicology, and phytochemistry of litchi fruit peel and seed. *J. Food Biochem*. 2021, 45(12), e13997. <https://doi.org/10.1111/jfbc.13997>
- Chukwuma, I., Mashele, S., Akuru, A. Evaluation of the *in vitro* α -amylase inhibitory, antiglycation, and antioxidant properties of *Punica granatum* L. (pomegranate) fruit peel acetone extract and its effect on glucose uptake and oxidative stress in hepatocytes. *J. Food Biochem*. 2020, 44, e13175.
- Chukwuma, I., Motlalepula, G., Mohammed, A., Ochuko, L., Matimbha, H., Shahidul I., Medicinal plants with concomitant anti-diabetic and anti-hypertensive effects as potential sources of dual acting therapies against diabetes and hypertension: A review. *J. Ethnopharmacol.*, 2019, 235, 329–360. <https://doi.org/10.1016/j.jep.02.024>.
- Chun, H., Henckel, M., Knaub, A., Hull, E., Pott, B., Ramirez, G., Reusch, E., Keller, A. C. (-)-Epicatechin Reverses Glucose Intolerance in Rats Housed at Thermoneutrality. *Planta medica*. 2022, 88(9-10), 735–744. <https://doi.org/10.1055/a-1843-9855>
- Coman, V., Teleky, B., Mitrea, L., Martău, G., Szabo, K., Călinoiu, L., Vodnar, D. Bioactive potential of fruit and vegetable wastes, Advances in food and nutrition research. *Elsevier*. 2020:157–225.
- Contreras-Castro, I., Oidor-Chan, H., Bustamante-Camilo, P., Pelayo-Zaldívar, C., Díaz de León-Sánchez, F., & Mendoza-Espinoza, A. Chemical Characterization and Evaluation of the Antihyperglycemic Effect of Lychee (*Litchi chinensis* Sonn.) cv. Brewster. *J. Med. Food*, 2022 25(1), 61–69. <https://doi.org/10.1089/jmf.2021.0098>
- Cowan, A., Wolstenholme, B. Avocado. In Encyclopedia of Food and Health; Caballero, B., Finglas, P.M., Toldrá, F., Eds.; Academic Press: Oxford, UK, 2016; pp. 294–300
- Cowie, C., Casagrande, S., Geiss, L. Prevalence and Incidence of Type 2 Diabetes and Prediabetes. In: Cowie CC, Casagrande SS, Menke A, Cissell MA, Eberhardt MS, Meigs JB, Gregg EW, Knowler WC, Barrett-Connor E, Becker DJ, Brancati FL, Boyko EJ, Herman WH, Howard BV, Narayan KMV, Rewers M, Fradkin JE, editors. Diabetes in America. 3rd ed. Bethesda (MD):

- National Institute of Diabetes and Digestive and Kidney Diseases (US); 2018; Aug. CHAPTER 3.
- Cremonini, E., Fraga, G., Oteiza PI. (–)-Epicatechin in the control of glucose homeostasis: Involvement of redox-regulated mechanisms. *Free Radic Biol Med.* 2019, 130, 478–488. <https://doi.org/10.1016/j.freeradbiomed.2018.11.010>
- Daiuto, É., Tremocoldi, M., Alencar, S., Vieites, R., Minarelli, P. Composição química e atividade antioxidante da polpa e resíduos de abacate ‘Hass’. *Rev. Bras. Frutic.* 2014, 36, 417–424
- Damanik, J., Yunir, E. Type 2 Diabetes Mellitus and Cognitive Impairment. *Acta Med Indones.* 2021; Apr;53(2):213-220.
- Dash, R., Babu, R., Srinivas, N. Reappraisal and perspectives of clinical drug-drug interaction potential of α -glucosidase inhibitors such as acarbose, voglibose, and miglitol in the treatment of type 2 diabetes mellitus. *Xenobiotica.* 2018;48(1):89-108.
- Davies, M., Aroda, V., Collins, B., Gabbay, R., Green, J., Maruthur, N., Rosas, S., Del Prato, S., Mathieu, C., Mingrone, G. Management of Hyperglycemia in Type 2 Diabetes, 2022. A Consensus Report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care* 2022; 45, 2753–2786
- de Almeida, M., Ferreira, J., Augusto-Obara, T., da Cruz, T., Arruda, H., Santos, V., J. Ramos, J., D. A. Botrel and Botrel. R. Can lychee reduce the adipose tissue mass in rats? *Braz. Arch. Biol. Technol.*, 2018, 61, e18160483.
- de Beer, D., Steyn, N., Joubert, E., Muller, N. Enhancing the polyphenol content of a red-fleshed Japanese plum (*Prunus salicina* Lindl.) nectar by incorporating a polyphenol-rich extract from the skins. *J Sci Food Agric.* 2012; Oct;92(13):2741-50. doi: 10.1002/jsfa.5704.
- de Ferranti, D., de Boer, H., Fonseca, V, Fox, S., Golden, H., Lavie, J., Magge, N., Marx, N., McGuire, K., Orchard, J., Zinman, B., Eckel, R. Type 1 diabetes mellitus and cardiovascular disease: a scientific statement from the American Heart Association and American Diabetes Association. *Diabetes Care.* 2014, 37:2843-63. 10.2337/dc14-1720
- Deacon, C. Dipeptidyl peptidase 4 inhibitors in the treatment of type 2 diabetes mellitus. *Nature Rev Endocrinol.* 2020;16(11):642- 653.
- DeFronzo, R., Inzucchi, S., Abdul-Ghani, M., Nissen, E. Pioglitazone: the forgotten, cost-effective cardioprotective drug for type 2 diabetes. *Diab Vasc Dis Res.* 2019;16(2):133-143.
- Derosa, G., & Maffioli, P. α -Glucosidase inhibitors and their use in clinical practice. *Arch. Med.* 2012, 8(5), 899–906. <https://doi.org/10.5114/aoms.2012.31621>

- Dimas, S., Lagou, V., Barker, A., Knowle, W., Mägi, R., Hivert, F., Benazzo, A., Rybin, D., Jackson, U., Stringham, M., Song, C., Fischer-Rosinsky, A., Boesgaard, W., Grarup, N., Abbasi, A., Assimes, L., Hao, K., Yang, X., Lecoecur, C., Barroso, I., Bonnycastle, L., Böttcher, Y., Bumpstead S, Chines PS, Erdos MR, Graessler J, Kovacs P, Morken MA, Narisu N, Payne F, Stancakova A, Swift AJ, Tönjes A, Bornstein, R., Cauchi, S., Froguel, P., Meyre, D., Schwarz, E., Häring, U., Smith U, Boehnke, M., Bergman, N., Collins FS, Mohlke, L., Tuomilehto, J., Quertemous T, Lind L, Hansen, T., Pedersen, O., Walker, M., Pfeiffer AF, Spranger J, Stumvoll M, Meigs, B., Wareham, J., Kuusisto, J., Laakso, M., Langenberg, C., Dupuis, J., Watanabe, M., Florez JC, Ingelsson E, McCarthy MI, Prokopenko, I. MAGIC Investigators. Impact of type 2 diabetes susceptibility variants on quantitative glycemic traits reveals mechanistic heterogeneity. *Diabetes*. 2014 Jun;63(6):2158-71. doi: 10.2337/db13-0949.
- Ding, C., Tang, Y., Zhu, W., Huang, P., Lian, P., Ran, J., Huang, X. Sodium-glucose cotransporter protein-2 inhibitors and glucagon-like peptide-1 receptor agonists versus thiazolidinediones for nonalcoholic fatty liver disease: A network meta-analysis. *Acta Diabetol*. 2022, 59, 519–533.
- Dirir, M., Daou, M., Yousef, F., Yousef, F. A review of alpha-glucosidase inhibitors from plants as potential candidates for the treatment of type-2 diabetes. *Phytochemistry reviews: proceedings of the Phytochem Lett*. 2022, 21(4), 1049–1079. <https://doi.org/10.1007/s11101-021-09773-1>
- Donnarumma, G., Buommino, E., Baroni, A., Auricchio, L., De Filippis, A., Cozza, V., Msika, P., Piccardi, N., Tufano, A. Effects of AV119, natural sugar from avocado, on *Malassezia furfur* invasiveness and on the expression of HBD-2 and cytokines in human keratinocytes. *Exp Dermatol* 2007; 16(11):912–919. <https://doi.org/10.1111/j.1600-0625.2007.00613.x>
- Dreher, L., Davenport, J. Hass avocado composition and potential health effects. *Crit. Rev. Food Sci. Nutr*. 2013, 53, 738–750.
- Drucker, J., and Nauck, A. The incretin system: Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 2006; 1696–1705. doi:10.1016/S0140-6736(06)69705-5
- Duda-Chodak, A., Tarko, T. and Tuszyński, T. Antioxidant activity of apples – an impact of maturity stage and fruit part. *Acta Sci. Pol. Technol. Aliment.*, 2011; 10(4), 443–454.
- Eckel, H., Kahn, E., Ferrannini, E, Goldfine, B., Nathan, M., Schwartz, W, Smith, J., Smith, R. Obesity and type 2 diabetes: what can be unified and what needs to be individualized? *J Clin Endocrinol Metab*. 2011;96(6):1654–63.
- Eftekharpour, E., Fernyhough, P. Oxidative stress and mitochondrial dysfunction associated with peripheral neuropathy in type 1 diabetes. *Antioxid Redox Signal* 2022. 37:578–596.

- Egbonu, A., Cemaluk, C., Some Antinutrient Compositions and in Vitro Antioxidant Properties of Milled Carica Papaya (Pawpaw) Peels and Seeds. *Applied Science Reports*, 2018 Vol. 17, No. 3, 2017, Available at SSRN: <https://ssrn.com/abstract=3200914> or <http://dx.doi.org/10.2139/ssrn.3200914>
- Eguchi, N., Vaziri, D., Dafoe, C., Ichii, H. The Role of Oxidative Stress in Pancreatic β Cell Dysfunction in Diabetes. *Int. J. Mol.Sci.* 2021; 22, 1509. <https://doi.org/10.3390/ijms22041509>
- Ekoru, K., Ayo P., Doumatey, A., Bentley, R., Guanjie, C., Jié, Z., Daniel, S., Olufemi, F., Godfrey O., Benjamin, E., Kofi, A., Jokotade, A., Williams, B., Albert, A., Joseph, A., Thomas, J., Johnnie O., Clement, A., Francis, C., Georgia, D., Adebawale, A., and Charles, R. “Type 2 diabetes complications and comorbidity in Sub-Saharan Africans.” *E Clinical Medicine* 16. 2019: 30 - 41.
- El-razek ABD, H. NMR Assignments of Four Catechin Epimers. *Asian J. Chem.* 2007, 19(6), 4867 – 4872.
- ElSayed, A., Aleppo, G., Aroda, R., Bannuru, R., Brown, M., Bruemmer, D. Classification and Diagnosis of Diabetes: Standards of Care in Diabetes-2023. *Diabetes Care*. 2023;46:S19–S40.
- Erdin, Z., and Aydın, Ç. “Type 2 diabetes mellitus: current diagnosis and treatment.” *J. Transl. Med.* 2023; n. pag.
- Erukainure, O.L., Ajiboye, J.A., Adejobi R.A., Okafor, O.Y., Adenekan, S.O. Protective effect of pineapple (*Ananas cosmosus*) peel extract on alcohol-induced oxidative stress in brain tissues of male albino rats, *Asian Pac. J. Trop. Dis.* 2011, 1(1), 5-9, 2222-1808. [https://doi.org/10.1016/S2222-1808\(11\)60002-9](https://doi.org/10.1016/S2222-1808(11)60002-9)
- Erzse, A., Stacey, N., Chola, L., Tugendhaft, A., Freeman M, Hofman K. The direct medical cost of type 2 diabetes mellitus in South Africa: a cost of illness study. *Glob Health Action*. 2019;12(1):1636611.
- Esparza, I., Jiménez-Moreno, N., Bimbela, F., Ancín-Azpilicueta, C., Gandía, L. Fruit and vegetable waste management: Conventional and emerging approaches. *J. Environ. Manag.* 2020; 265, 110510 doi: 10.3390/molecules28062631.
- Evans, L., Goldfine, D., Maddux, A., Grodsky, M. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes*. 2003;52(1):1–8. doi: 10.2337/diabetes.52.1.1.

- Faselis, C., Katsimardou, A., Imprialos, K., Deligkaris, P., Kallistratos, M., Dimitriadis, K. Microvascular Complications of Type 2 Diabetes Mellitus. *Curr Vasc Pharmacol.* 2020;18(2):117-124. doi: 10.2174/1570161117666190502103733. PMID: 31057114.
- Feng, G., Hong-Lan, L., Shen, Q., Zhuo-Ying, L., Xiaowei, J., and Yong-Bing, X. "Population attributable risk of excess weight, abdominal obesity, and physical inactivity for type 2 diabetes in Chinese men and women." *Ann. Transl. Med.* 9 2021: n. pag.
- Ferguson, D., Sattar, N. Reducing cardiovascular disease risk in type 2 diabetes: is the focus on glycaemia warranted? *Diabetes Obes Metab.* 2013, 15:387-91. 10.1111/dom.12018
- Ferreira, M., Falé, Z., Santos, L. Sustainability in skin care: Incorporation of avocado peel extracts in topical formulations. *Molecules* 2022, 27, 1782.
- Figueroa, G., Borrás-Linares, I., Lozano-Sánchez, J., Segura-Carretero, A. Comprehensive Identification of Bioactive Compounds of Avocado Peel by Liquid Chromatography Coupled to Ultra-High-Definition Accurate-Mass Q-TOF. *Food Chem.* 2018, 245, 707–716.
- Filion, B., Douros, A., Azoulay, L., Yin, H., Yu, O. Suissa, S. Sulfonylureas as initial treatment for type 2 diabetes and the risk of adverse cardiovascular events: A population-based cohort study. *Br. J. Clin. Pharmacol.* 2019; 85, 2378–2389.
- Francis, D., Celestine, A., Choice, N., Okorie, P., Igwe, U., Jide, U., Princewill, U., Pamela Anyaeji and Nwachukwu Daniel. "The Effect of Ethanolic Seed Extract of Citrullus lanatus (Watermelon) on Blood Glucose Level and Lipid Profile of Diabetic Wistar Rats." *Eur. J. Med. Plant* 2019; n. pag.
- Fu, Y., Li, S., Lin, M., Qiao, Y., Yang, R., Li, M., Dong, B., Xiang, P., Zheng, Q., Lu, L., Yuan, B., Ye, H., & Liang, R. Antidiabetic effects of tea. *Molecules* 2017; 22(5), 849. <https://doi.org/10.3390/molecules22050849>
- Fulgoni, L., Dreher, M., Davenport, J. Avocado consumption is associated with better diet quality and nutrient intake, and lower metabolic syndrome risk in US adults: Results from the National Health and Nutrition Examination Survey (NHANES) 2001-2008. *Nutr. J.* 2013, 12, 1
- Galano, A., Alvarez-Idaboy, A computational methodology for accurate predictions of rate constants in solution: Application to the assessment of primary antioxidant activity. *J. Comput. Chem.* 2013, 34, 2430–2445.
- Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, B., Ostolaza, H., Martín, C. Pathophysiology of Type 2 Diabetes Mellitus. *Int. J. Mol. Sci.* 2020, 21(17), 6275. <https://doi.org/10.3390/ijms21176275>

- Gao, X., Wang, M., Liu, C., and Zhang, M. “[Research on the association between physical activity and type 2 diabetes].” *Zhonghua liu xing bing xue za zhi = Zhonghua liuxingbingxue zazhi* 43 10. 2022; 1681-1684.
- Ghimire, P., Dhamoon, S. Ketoacidosis. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK534848/>
- Giacca, A., Solini, A., Frontoni, S. Del Prato, S. Metformin Benefits: Another Example for Alternative Energy Substrate Mechanism? *Diabetes Care* 2021, 44, 647–654
- Gilbert, P., Pratley R. GLP-1 analogs and DPP-4 inhibitors in type 2 diabetes therapy: review of head-to-head clinical trials *Front. Endocrinol.*, 11 2020; p. 178
- Glezeva, N., Master C., Kenneth M., Mark L., Joe G., and Chris, W. “Diabetes and complications of the heart in Sub-Saharan Africa: An urgent need for improved awareness, diagnostics and management.” *Diabetes Res Clin Pract* 137. 2018: 10-19
- Golden, H., Chittaranjan, Y., Sanat, P., Robert, H., and William, K. “Racial/ethnic differences in the burden of type 2 diabetes over the life course: a focus on the USA and India.” *Diabetologia*. 2019: 1-10.
- Gondi, M., Prasada, J. Ethanol extract of mango (*Mangifera indica L.*) peel inhibits α -amylase and α -glucosidase activities and ameliorates diabetes-related biochemical parameters in streptozotocin (STZ)-induced diabetic rats. *J Food Sci Technol*. 2015 Dec;52(12):7883-93. doi: 10.1007/s13197-015-1963-4.
- Gong, Y., Fang, F., Zhang, X., Liu, B., Luo, H., Li, Z., Zhang, X., Zhang, Z., & Pang, X. B type and complex A/B type epicatechin trimers isolated from Litchi pericarp aqueous extract show high antioxidant and anticancer activity. *Int. J. Mol. Sci.*, 2018 19(1), 301. <https://doi.org/10.3390/ijms19010301>
- Gonzalez, D., Lee, M., Marchetti, M., Pietropaolo, M., Towns, R., Vaccaro, M., Watada, H., Wiley, J. The emerging role of autophagy in the pathophysiology of diabetes mellitus, 7(1) 2011; 2-11.
- Goyal, R., Singhal, M., Jialal I. Type 2 Diabetes. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan Available from: <https://www.ncbi.nlm.nih.gov/books/NBK513253/>
- Goyal, Yamini, Amit Kumar Verma, Prakash Chandra Joshi and Kapil Dev. “Contemplating the role of genetic variants of HHEX, CDKAL1, WFS1 and SLC30A8 genes of TYPE-2 diabetes in Asians ethnic groups.” *Gene Reports* 17. 2019; 100465.

- Guardado-Mendoza, R., Prioletta, A., Jiménez-Ceja, M., Sosale, A., Folli, F. The role of nateglinide and repaglinide, derivatives of meglitinide, in the treatment of type 2 diabetes mellitus. *Arch. Med. Sci.* 2013; 9, 936–943.
- Guo, J., Li, L., Pan, J., Qiu, G., Li, A., Huang, G., Xu, L. Pharmacological mechanism of Semen Litchi on antagonizing insulin resistance in rats with type 2 diabetes. *J Chinese Med. Material.* 2004; 27(6):435-
- Guoxiang, J., Sen, L., Lingrong, W., Yueming, J., Mouming, Z., Feng, C., Nagendra, P., Xuewu D., Bao, Y. Identification of a novel phenolic compound in litchi (*Litchi chinensis* Sonn.) pericarp and bioactivity evaluation, *Food Chem.* Volume 136, Issue 2, 2013, Pages 563-568, <https://doi.org/10.1016/j.foodchem.2012.08.089>.
- Gylfadottir, S., Weerachoenkul, D., Andersen, S., Niruthisard, S., Suwanwalaikorn, S., Jensen, S. Painful and non-painful diabetic polyneuropathy: Clinical characteristics and diagnostic issues. *J Diabetes Investig.* 2019;10(5):1148–57
- Hameister, R., Kaur, C., Dheen, T., Lohmann, H., Singh, G. Reactive oxygen/nitrogen species (ROS/RNS) and oxidative stress in arthroplasty. *J. Biomed. Mater. Res.* 2020, 108, 2073–2087.
- Hansen, D., Dendale, P., Beelen, M. Plasma adipokine and inflammatory marker concentrations are altered in obese, as opposed to non-obese, type 2 diabetes patients. *Eur J Appl Physiol* 109, 397–404. 2010. <https://doi.org/10.1007/s00421-010-1362-5>
- Harborne, B. The flavonoids: advances in research since 1980, 2013.
- Heerspink, L., Stefánsson, V., Correa-Rotter, R., Chertow, M., Greene, T., Hou, F., Mann, E., McMurray, V., Lindberg, M., Rossing, P. Dapagliflozin in Patients with Chronic Kidney Disease. *N. Engl. J. Med.* 2020; 383, 1436–1446
- Henríquez, A., Sergio F. Almonacid, C., Tania, V., Manuel A., Lorena C., Ricardo S., and Hernán S. “Determination of antioxidant capacity, total phenolic content and mineral composition of different fruit tissue of five apple cultivars grown in Chile.” *Chil. J. Agric. Res.* 70. 2010: 523-536.
- Hirasawa, M., Shimura, K., Shimizu, A., Mura, K., Tokue, C., Arai, S. Quantification and functional analysis of dietary fiber and polyphenols in avocado. *J. Jpn. Soc. Food Sci. Technol.* 2008, 55, 95–101.
- Hossain, U., Das, K., Ghosh S, Sil, C. An overview on the role of bioactive α -glucosidase inhibitors in ameliorating diabetic complications. *Food Chem Toxicol.* 2020; 145:111738.

- Hsu, Y., & Wen, H. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.* 2002, 277(25), 22131–22139. <https://doi.org/10.1074/jbc.M111883200>
- Hu, B. Globalization of diabetes: the role of diet, lifestyle, and genes. *Diabetes Care.* 2011 Jun;34(6):1249-57. doi: 10.2337/dc11-0442.
- Huang, J., Xie, M., He, L., Song, X., Cao, T. Chlorogenic acid: A review on its mechanisms of anti-inflammation, disease treatment, and related delivery systems. *Front. Pharmacol.* 2023, 14, 1218015.
- Huang, T., Li W., Mei B., Jianwen Z., Dongya Y., Yongjun H., Yuhe W., Tianbo J., and Wei C. “Influence of IGF2BP2, HMG20A, and HNF1B genetic polymorphisms on the susceptibility to Type 2 diabetes mellitus in Chinese Han population,” *Bioscience Reports* 40. 2020: n. pag
- Hurrle, S., and Hsu, W. “The etiology of oxidative stress in insulin resistance,” *Biomed. J.*, vol. 40, no. 5, pp. 257–262, 2017
- Hurtado-Fernandez, E., Carrasco-Pancorbo, A., Fernandez-Gutierrez, A. Profiling LC-DAD-ESI-TOF MS method for the determination of phenolic metabolites from avocado (*Persea americana*). *J. Agric. Food Chem.* 2011, 59, 2255–2267
- Hussain, H., Mamadalieva, Z., Hussain, A., Hassan, U., Rabnawaz, A., Ahmed, I., Green, R. Fruit Peels: Food Waste as a Valuable Source of Bioactive Natural Products for Drug Discovery. *Curr. Issues Mol. Biol.* 2022, 44, 1960–1994.
- Ibrahim, R., and Mohamed, G. Litchi chinensis: medicinal uses, phytochemistry, and pharmacology, *J. Ethnopharmacol.*, 2015, 174, 492–513
- Imamura, M., Shigemizu, D., Tsunoda, T, Assessing the clinical utility of a genetic risk score constructed using 49 susceptibility alleles for type 2 diabetes in a Japanese population. *J Clin Endocrin Metabol.* 2013;98:E1667-E1673.
- International Diabetes Federation (IDF), South Africa Diabetes 2021 Report 2000–2045. 2023. <https://diabetesatlas.org/data/en/country/185/za.html>. Accessed 7 Aug 2023.
- International Diabetes Federation. IDF Diabetes Atlas, 10th edn. 2021, https://diabetesatlas.org/idfawp/resource-files/2021/07/IDF_Atlas_10th_Edition_2021.pdf (Accessed 2 January 2022).
- Isabelle, S., Vanessa, C., Bárbara, G., Laís de Oliveira, S., Vanessa, P., Lourdes, C., Suely, F., Alexandre, T. Hass avocado (*Persea americana* Mill.) oil enriched in phenolic compounds and tocopherols by expeller-pressing the unpeeled microwave-dried fruit, *Food Chem.* Volume 286, 2019, Pages 354-361. <https://doi.org/10.1016/j.foodchem.2019.02.014>

- Javed, S., Petropoulos, N., Alam, U., Malik, A. Treatment of painful diabetic neuropathy. *Ther Adv Chronic Dis.* 2015 Jan;6(1):15-28. doi: 10.1177/2040622314552071.
- Jha, N., Ryu, J., Choi, H., Kaushik, K. Generation and role of reactive oxygen and nitrogen species induced by plasma, lasers, chemical agents, and other systems in dentistry. *Oxidative Med. Cell. Longev.* 2017, 2017, 7542540.
- Jibril, M., Abdul-Hamid, A., Ghazali, M., Dek, P., Ramli, S., Jaafar, H., et al. Antidiabetic antioxidant and phytochemical profile of yellow-fleshed seeded watermelon (*Citrullus lanatus*) extracts. *J food nutr res*, 2019; 7(1), 82–95. <https://doi.org/10.12691/jfnr-7-1-10>
- Jomova, K., Raptova, R., Alomar, Y., Alwasel, H., Nepovimova, E., Kuca, K., Valko, M. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. *Arch. Toxicol.* 2023, 97, 2499–2574.
- Jung, K., Sang, K., Chang-Gu, H., Nam, L. Antioxidant Chemical Constituents from the Stems of *Cleyera japonica* Thunberg. *Int. J. Pharmacol.* 2012, 8(5), 410–415. DOI: 10.3923/ijp.2012.410.415
- Kadouh, C., Sun, S., Zhu, W., Zhou, K. α -Glucosidase inhibiting activity and bioactive compounds of six red wine grape pomace extracts. *J Funct Foods.* 2016 Oct; 26:577-584. doi: 10.1016/j.jff.2016.08.022. Epub 2016 Aug 26.
- Kainsa, S., Singh, R. Flavan-3-ol Isomers Isolated from *Euphorbia Thymifolia* Linn. *Phcog Commn.* 2016, 6(1), 28–33. DOI:10.5530/pc.2016.1.5
- Kario, K., Ferdinand, C., O’Keefe, H. Control of 24-hour blood pressure with SGLT2 inhibitors to prevent cardiovascular disease. *Progr Cardiovascu Dis.* 2020 doi: 10.1016/j.pcad.2020.04.003.
- Kashtoh H., Baek H. Recent Updates on Phytoconstituent Alpha-Glucosidase Inhibitors: An Approach towards the Treatment of Type Two Diabetes. *Plants.* 2022, 11(20):2722. <https://doi.org/10.3390/plants11202722>
- Katherina, F., Javiera, L., Simulated digestion of proanthocyanidins in grape skin and seed extracts and the effects of digestion on the angiotensin I-converting enzyme (ACE) inhibitory activity, *Food Chem.*, Volume 139, Issues 1–4, 2013, Pages 196-202, <https://doi.org/10.1016/j.foodchem.2013.01.021>
- Katja, G., Suryanto E., Wehantouw, F. The Potential of Avocado Leaves (*Persea Americana* Mill) as a Source of Natural Antioxidants. *Chem Prog.* 2019; 2:58–64.
- Kaur, M., Misra, S., Swarnkar, P., Patel, P., Das Kurmi, B., Das Gupta, G., Singh, A. Understanding the role of hyperglycemia and the molecular mechanism associated with diabetic neuropathy and

- possible therapeutic strategies. *Biochem Pharmacol.* 2023 Sep; 215:115723. doi: 10.1016/j.bcp.2023.115723.
- Kawamori, Y., Kajimoto, H., Kaneto, Y., Umayahara, Y., Fujitani, T., Miyatsuka, H., Watada, B., Leibiger, Y., Yamasaki, M. Oxidative stress induces nucleo-cytoplasmic translocation of pancreatic transcription factor PDX-1 through activation of c-Jun NH(2)-terminal kinase, *Diabetes* 52(12) 2003; 2896-904
- Kernan, N., Viscoli, M., Furie, L., Young, H., Inzucchi, E., Gorman, M., Guarino, D., Lovejoy, A.M.; Peduzzi, P.N.; Conwit, R.; et al. Pioglitazone after Ischemic Stroke or Transient Ischemic Attack. *New Engl. J. Med.* 2016, 374, 1321–1331.
- Kessy, H. N. E., Wang, K., Zhao, L., Zhou, M., & Hu, Z. Enrichment and biotransformation of phenolic compounds from litchi pericarps with angiotensin I-converting enzyme (ACE) inhibition activity. *Food Sci. Technol.*, 2018 87, 301–309. <https://doi.org/10.1016/j.lwt.2017.09.003>
- Khan, M., B., Muhammad, H., Jeffrey, K., Romona, G., Halla, M., and Juma, K. “Epidemiology of Type 2 Diabetes – Global Burden of Disease and Forecasted Trends.” *J. Epidem. Glo. H.* 10. 2019: 107 - 111.
- Khan, N., le Roes-Hill, M., Welz, J., Grandin, A., Kudanga, T., Van Dyk S. Fruit waste streams in South Africa and their potential role in developing a bio-economy. *S Afr J Sci.* 2015;111(5/6), Art. #2014-0189, 11 pages. <http://dx.doi.org/10.17159/sajs.2015/20140189>
- Kim, J. Kim, Y., Fillmore, J. “Prevention of fat-induced insulin resistance by salicylate,” *J. of Clin. Investig.*, vol. 108, no. 3, pp. 437–446, 2001.
- Kirkman, S., Briscoe, S., Clark, N., Florez, H., Haas, B., Halter, B., Huang, S., Korytkowski, T., Munshi, N., Odegaard, S. Diabetes in older adults. *Diabetes Care.* 2012, 35, 2650–2664.
- Koch, T., Nakhoul, R., Nakhoul, F. Autophagy in diabetic nephropathy: a review. *Int Urol Nephrol* 52, 1705–1712.2020; <https://doi.org/10.1007/s11255-020-02545-4>
- Kosińska, A., Karamač, M., Estrella, I., Hernández, T., Bartolomé, B., Dykes, A. Phenolic Compound Profiles and Antioxidant Capacity of *Persea americana* Mill. Peels and Seeds of Two Varieties. *J. Agric. Food Chem.* 2012, 60, 4613–4619
- Kosińska, A., Karamač, M., Estrella, I., Hernández, T., Bartolomé, B., Dykes, G.A. Phenolic compound profiles and antioxidant capacity of *Persea americana* Mill. peels and seeds of two varieties. *J. Agric. Food Chem.*, 2012; 60(18), 4613-4619. <https://doi.org/10.1021/jf300090p>

- Kurek, M., Hlupić, L., Garofulić, E., Descours, E., Šćetar, M., Galić K. Comparison of protective supports and antioxidative capacity of two bio-based films with revalorized fruit pomaces extracted from blueberry and red grape skin. *Food Package*. 2019, 20, 100315.
- Kusuhara, S., Yoko, F., Shuntaro, O., Naomi, I., and Akiyoshi, U. “Pathophysiology of Diabetic Retinopathy: The Old and the New.” *Diabetes Metab J* 42 2018; 364 - 376.
- Larson-Meyer, E., Newcomer, R., Ravussin, E., Volaufova, J., Bennett, B., Chalew, S. Intrahepatic and intramyocellular lipids are determinants of insulin resistance in prepubertal children. *Diabetologia*. 2011;54(4):869–75
- Lee, E., & Park, S. The Emerging Roles of Antioxidant Enzymes by Dietary Phytochemicals in Vascular Diseases. *Life*. 2021. 11(3), 199. <https://doi.org/10.3390/life11030199>
- Lei, Z. Frontiers in Understanding the Pathological Mechanism of Diabetic Retinopathy. e-ISSN 1643-3750 *Med Sci Monit*, 2023; 29: e939658 DOI: 10.12659/MSM.93965
- Lenzen, S. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia*, 2008 51(2), 216–226. <https://doi.org/10.1007/s00125-007-0886-7>
- Levetan, C. Oral antidiabetic agents in type 2 diabetes. *Curr. Med. Res. Opin.* 2007; 23, 945–952
- Li, C. Chinese and related North American herbs phytopharmacology and therapeutic values (2nd ed., pp. 1–597). 2009; Boca Raton, London: CRC Press Taylor & Francis Group
- Li, J., Jiang, Y. Litchi Flavonoids: Isolation, Identification and Biological Activity. *Molecules*. 2007, 12(4), 745–758. <https://doi.org/10.3390/12040745>
- Li, Q., Liao, B., Li, H., Guo, W., Qu, L., & Li, M. Effect and mechanism of Litchi semen effective constituents on insulin resistance in rats with type 2 diabetes mellitus. *Zhong Yao Cai*, 2015; 38(7), 1466–1471
- Li, W., Liang, H., Zhang, W., Zhang, F., Deng, Y., Wei, C., Zhang, Y., Tang, J. Phenolic Profiles and Antioxidant Activity of Litchi (*Litchi Chinensis* Sonn.) Fruit Pericarp from Different Commercially Available Cultivars. *Molecules* 2012, 17, 14954-14967. <https://doi.org/10.3390/molecules171214954>
- Li, Y., Shang, S., Fei, Y., Chen, C., Jiang Y, Dang, L. Interactive relations of type 2 diabetes and abdominal obesity to cognitive impairment: A cross-sectional study in rural area of Xi'an in China. *J Diabetes Complicat*. 2018. 32:48–55. doi: 10.1016/j.jdiacomp.2017.09.006
- Liang, N., Kitts, D. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients* 2015, 8, 16.

- Ligthart, S., Hasbani, R., Ahmadizar, F., Genetic susceptibility, obesity and lifetime risk of type 2 diabetes: The ARIC study and Rotterdam Study. *Diabet Med.* 2021;38:e14639. <https://doi.org/10.1111/dme.14639>
- Lin, C., Chung, Y., & Hsu, C. Anti-cancer potential of litchi seed extract. *World J. Exp. Med.*, 2013 3, 56–61. <https://doi.org/10.5493/wjem.v3.i4.56>
- Liu, D., Tiantian, B., and Shenghe, J. “Mechanisms and Research Progress of Type 2 Diabetes Mellitus and Its Hepatic Complications.” *MEDS Clinical Medicine* 2023; n. pag.
- Lo, S., Russel, C., and Taylor, W. Determination of glycogen in small tissue samples. *J. Appl. Physiol.*, 1970. 28, 234–236
- Long, N., Dagogo-Jack S. Comorbidities of diabetes and hypertension: mechanisms and approach to target organ protection. *J Clin Hypertens* 2011; 13: 244–251.
- Long, N., Le Gresley, A., Wren, P. Thiazolidinediones: An In-Depth Study of Their Synthesis and Application to Medicinal Chemistry in the Treatment of Diabetes Mellitus. *Chem Med Chem* 2021; 16, 1717–1736
- López-Cobo, A., Gómez-Caravaca, M., Pasini, F., Caboni, F., Segura-Carretero, A., Fernández-Gutiérrez, A. HPLC-DAD-ESI-QTOF-MS and HPLC-FLD-MS as valuable tools for the determination of phenolic and other polar compounds in the edible part and by-products of avocado. *Lwt Food Sci. Technol.* 2016, 73, 505–513
- Lu, Y., Zhang, Y., Wang, Y., Wang, D., Lee, P., Gao, K., Byrns, R., Heber, D. California Hass avocado: Profiling of carotenoids, tocopherol, fatty acid, and fat content during maturation and from different growing areas. *J. Agric. Food Chem.* 2009, 57, 10408–10413
- Lv, W., Wang, X., Xu, Q., Lu, W. Mechanisms and Characteristics of Sulfonylureas and Glinides. *Curr. Top. Med. Chem.* 2020, 20, 37–56.
- Lv, Y., Yao, X., Li, X., Ouyang, Y., Fan, C., Qian, Y. Cell metabolism pathways involved in the pathophysiological changes of diabetic peripheral neuropathy. *Neural Regen. Res.* 19(3):p 598-605, 2024. | DOI: 10.4103/1673-5374.380872
- Lyu, X., Agar, T., Barrow, J., Dunshea, R., Suleria, R. Phenolic compounds profiling and their antioxidant capacity in the peel, pulp, and seed of Australian grown avocado. *Antioxidants* 2023, 12, 185.
- Lyu, X., Osman, A., Colin James Barrow, Frank Rowland Dunshea, and Hafiz Ansar Rasul Suleria. “Phenolic Compounds Profiling and Their Antioxidant Capacity in the Peel, Pulp, and Seed of Australian Grown Avocado.” *Antioxidants* 12 2023; n. pag.

- Ma, Q., Xie, H., Jiang, Y., & Wei, X. Phenolics and sesquiterpenes from litchi pericarp. *J. Funct. Foods*, 2014; 9, 156–161. <https://doi.org/10.1016/j.jff.2014.04.020>
- Mackin, G. Currie DPP-4 inhibitors *Diabetes Drug Notes* 2022, pp. 67-94
- Maddux, A., See, W., Lawrence, Jr., Goldfine, L., Goldfine, D., Evans, L. Protection against oxidative stress—induced insulin resistance in rat L6 muscle cells by micromolar concentrations of α -lipoic acid. *Diabetes*. 2001;50(2):404–410. doi: 10.2337/diabetes.50.2.404.
- Maggiore, U., Budde, K., Heemann U. Long-term risks of kidney living donation: review and position paper by the ERA-EDTA DESCARTES working group. *Nephrol Dial Transplant* 2017; 32: 216–223.
- Magwaza, S., Tesfay, Z. A Review of Destructive and Non-destructive Methods for Determining Avocado Fruit Maturity. *Food Bioprocess Technol* 8, 1995–2011 2015. <https://doi.org/10.1007/s11947-015-1568-y>
- Maha, A., Wafa, A., Eman, I., Manal, E. Anti-oxidant, anti-inflammatory and anti-cancer activities of avocado (*Persea americana*) fruit and seed extract, Journal of King Saud University - Science, Volume 31, Issue 4, 2019, Pages 1358-1362, <https://doi.org/10.1016/j.jksus.2018.10.010>.
- Mahajan, A., Taliun, D., Thurner, M. Fine-mapping type 2 diabetes loci to single-variant resolution using high-density imputation and islet-specific epigenome maps. *Nat Genet*. 2018; 50:1505-1513
- Mahmoud, H., Abdel-Hady, A., and Badawi, M. Hypocholesterolemic Effect of Antioxidant Containing Fruits in Rats Fed on High-Cholesterol and Fat Diet. *Res. J. Appl. Sci.*, 2013; 9, 4233-4244.
- Majety, P., Lozada, A., Edem, D., Hamdy, O. Pharmacological approaches to the prevention of type 2 diabetes mellitus. *Front Endocrinol* 2023 Mar 9; 14:1118848. doi: 10.3389/fendo.2023;1118848.
- Man, S., Ma, J., Yao, J., Cui, J., Wang, C., Li, Y., Ma, L., & Lu, F. Systemic perturbations of key metabolites in type 2 diabetic rats treated by polyphenol extracts from *Litchi chinensis* seeds. *J. Agric. Food Chem.*, 2017; 65(35), 7698–7704
- M., Sarkar, M., Khan, A., Biswas, M., Masi, A., Rakwal, R., Agrawal, K., Srivastava, A., Sarkar, A. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plants— maintenance of structural individuality and functional blend. *Adv. Red. Res.* 2022, 5, 100039.
- Marrazzo, P., Angeloni, C., Freschi, M., Lorenzini, A., Prata, C., Maraldi, T., Hrelia, S. Combination of epigallocatechin gallate and sulforaphane counteracts in vitro oxidative stress and delays stemness loss of amniotic fluid stem cells. *Oxidative Med. Cell. Longev*. 2018, 2018, 30647811.

- Mashile, B., Setlhodi, R.; Izu, O., Erukainure, L., Mashele, S., Makhafola, J., Eze, C., Chukwuma, J.I. Temperature-dependent extraction and chromatographic recovery and characterization of ellagitannins with potent antioxidant and glycaemic control properties from “Wonderful” pomegranate peel. *Int J Food Sci Tech*. 2024, 59, 408–424. <https://doi.org/10.1111/ijfs.16823>
- Matowane, R., Mashele, S., Makhafola, J., Chukwuma, I. The ameliorative effect of zinc acetate with caffeic acid in the animal model of type 2 diabetes. *Biomed. Pharmacother*. 2023 163:114779. <https://doi.org/10.1016/j.biopha.2023.114779>
- Mayosi, M., Flisher, J., Lalloo, G., Sitas, F., Tollman, M., Bradshaw, D. The burden of non-communicable diseases in South Africa. *Lancet* 2009, 374, 934–947.
- Mazidi, M., Rezaie, P., Gao, K., Kengne, P. Effect of sodium-glucose cotransport-2 inhibitors on blood pressure in people with type 2 diabetes mellitus: a systematic review and meta-analysis of 43 randomized control trials with 22 528 patients. *J Am Heart Assoc*. 2017;6(6):e004007.
- Mehra, R., Kumar, H., Kumar, N., Kaushik, R. Red rice conjugated with barley and rhododendron extracts for new variant of beer. *J Food Sci Technol* 2020.
- Melgar, B., Dias, I., Ciric, A., Sokovic, M., Garcia-Castello, M., Rodrigues-Lopez, D., Barros, L., Ferreira, F. Bioactive characterization of *Persea americana* Mill. by-products: A rich source of inherent antioxidants. *Ind Crops*, 2018; 111, 212-218. <https://doi.org/10.1016/j.indcrop.2017.10.024>
- Mende, W. Chronic kidney disease and SGLT2 inhibitors: a review of the evolving treatment landscape. *Advances Therap*. 2022:1-17.
- Mfotie, E., Ndemangou, B., Akinyelu, J., Munvera, M., Chukwuma, I., Mkounga, P., Mashele, S., Makhafola, J., McGaw, J. *In vitro* antiproliferative, anti-inflammatory effects and molecular docking studies of natural compounds isolated from *Sarcocephalus pobeguini* (Hua ex Pobeg). *Front. Pharmacol*. 2023, 14, 1205414.
- Mhatre, V., Bhagat, A., & Yadav, P. Proteinaceous pancreatic lipase inhibitor from the seed of Litchi chinensis. *Food technol. Biotech.*, 2019; 57(1), 113–118
- Michalska, A., Wojdyło, A., Majerska, J., Lech, K., Brzezowska, J. Qualitative and Quantitative Evaluation of Heat-Induced Changes in Polyphenols and Antioxidant Capacity in *Prunus domestica* L. By-products. *Molecules*. 2019 Aug 19;24(16):3008. doi: 10.3390/molecules24163008.
- Mothibe, E., Sibanda, M. African Traditional Medicine: South African Perspective. In *Traditional and Complementary Medicine*; Mordeniz, C., Ed.; *IntechOpen*, Ltd.: London, UK, 2019.

- Motloun, M., Mashele, S., Matowane, R., Swain, S., Bonnet, L., Noreljaleel, M., Oyedemi, O., Chukwuma, I. Synthesis, characterization, antidiabetic and antioxidative evaluation of a novel Zn (II)-gallic acid complex with multi-facet activity. *J. Pharm. Pharmacol.* 2020, 72, 1412–1426.
- Mutyambizi, C., Pavlova, M., Hongoro, C. Inequalities and factors associated with adherence to diabetes self-care practices amongst patients at two public hospitals in Gauteng, South Africa. *BMC Endocr Disord* 2020. <https://doi.org/10.1186/s12902-020-0492-y>
- Ni, L., Yuan, C., Chen, G., Zhang, C., Wu, X. SGLT2i: beyond the glucose-lowering effect. *Cardiovasc Diabetol.* 2020 Jun 26;19(1):98. doi: 10.1186/s12933-020-01071-y.
- Niewczas, A., Pavkov, E., Skupien, J., Smiles, A., Md Dom, I., Wilson, M., Park, J., Nair, V., Schlafly, A., Saulnier, J. A signature of circulating inflammatory proteins and development of end-stage renal disease in diabetes. *Nat. Med.* 2019; 25, 805–813
- Nigro, N., Grossmann, M., Chiang, C., & Inder, J. Polyuria-polydipsia syndrome: a diagnostic challenge. *Intern. Med. J.* 2018 48(3), 244–253. <https://doi.org/10.1111/imj.13627>
- Nirmal, P., Anandu, K., Anjaly, M., Kartik, S., Karthik, B., Priyamvada, T., Fahad, A. and Ravi, P. “Valorization of Fruit Waste for Bioactive Compounds and Their Applications in the Food Industry.” *Foods* 2023; n. pag.
- Norton, L., Shannon, C., Gastaldelli, A., DeFronzo, A. Insulin: The master regulator of glucose metabolism. *Metabolism.* Apr;129:155142.*metabol.*2022; 155142.
- Nowotny, J., Hohn, A., Weber, G. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus, *Biomolecules.* 2015; 194–222, <https://doi.org/10.3390/biom5010194>
- Oboh, G., Ademosun, O. Phenolic extracts from grapefruit peels (*Citrus paradisi*) inhibit key enzymes linked to type-2 diabetes and hypertension. *J Food Biochem.* 2010; doi:10.1111/j.1745-4514.2010.00495.x
- Oboh, G., Odubanjo, O., Bello, F., Ademosun, O., Oyeleye, I., Nwanna, E., Ademiluyi, O. Aqueous extracts of avocado pear (*Persea americana* Mill.) leaves and seeds exhibit anti-cholinesterases and antioxidant activities in vitro. *J. Basic Clin. Physiol. Pharmacol.* 2016, 27, 131–140.
- Oguntibeju, O. Type 2 diabetes mellitus, oxidative stress, and inflammation: examining the links. *Int J Physiol Pathophysiol Pharmacol.* Jun 15;11(3):45-63. 2019
- Ogurtsova, K., Guariguata, L., Barengo, C., Ruiz, L., Sacre, W., Karuranga, S., Sun, H., Boyko, J., Magliano, J. IDF diabetes Atlas: Global estimates of undiagnosed diabetes in adults for 2021. *Diabetes Res Clin Pract.* 2022 Jan;183:109118. doi: 10.1016/j.diabres.2021.109118.

- Oshitari, T. “The Pathogenesis and Therapeutic Approaches of Diabetic Neuropathy in the Retina.” *Int. J. Mol. Sci.* 2021. 22
- Oynotkinova, S., Viktoria, K., Evgeny, E., and Gusarenko, S. “Assessment of the role of unhealthy diet and low physical activity in the development of diabetes mellitus and cardiovascular complications (literature review).” *HEALTH CARE OF THE RUSSIAN FEDERATION*: 2022.
- Padhi, S., Nayak, K., Behera, A. Type II diabetes mellitus: a review on recent drug-based therapeutics. *Biomed Pharmacother.* 2020 Nov;131:110708. doi: 10.1016/j.biopha.2020;110708.
- Pahua-Ramos, E., Ortiz-Moreno, A., Chamorro-Cevallos, G., Hernandez-Navarro, D., Garduno-Siciliano, L., Necoechea-Mondragon, H., Hernandez-Ortega, M. Hypolipidemic effect of avocado (*Persea americana* Mill) seed in a hypercholesterolemic mouse model. *Plant Foods Hum. Nutr.* 2012, 67, 10–16
- Palmieri, M., McGinity, C., Wink, A., McVicar, W. Nitric oxide in macrophage immunometabolism: Hiding in plain sight. *Metabolites* 2020, 10, 429.
- Pang, L., Lian, X., Liu, H., Zhang, Y., Li, Q., Cai, Y., Ma, H., Yu, X. Understanding diabetic neuropathy: focus on oxidative stress. *Oxid Med Cell Longev* 2020:9524635.
- Papachristoforou V., Lambadiari, M., Makrilakis, K. Association of Glycemic Indices (Hyperglycemia, Glucose Variability, and Hypoglycemia) with Oxidative Stress and Diabetic Complications, *J Diabetes Res* 2020. 7489795.
- Park, H., Park, H., Hong, G., Lee, S., Baek, H., Lee, I., Heo, W., & Yokozawa, T. Oligonol, a low-molecular-weight polyphenol derived from lychee peel, attenuates diabetes-induced pancreatic damage by inhibiting inflammatory responses via oxidative stress-dependent mitogen-activated protein kinase/nuclear factor-kappa B signaling. *Phytother. Res.*, 2018; 32(12), 2541–2550. <https://doi.org/10.1002/ptr.6194>
- Patanè, T., Putaggio, S., Tellone, E., Barreca, D., Ficarra, S., Maffei, C., Calderaro, A., & Laganà, G. Catechins and Proanthocyanidins Involvement in Metabolic Syndrome. *Int. J. Mol. Sci.* 2023 24(11), 9228. <https://doi.org/10.3390/ijms24119228>
- Paul, S., Azam, A., and Rajesh, G Katare. “Molecular complexities underlying the vascular complications of diabetes mellitus - A comprehensive review.” *J Diabetes Complications* 2020: 107613.
- Pazarín-Villaseñor, L., García-Salas, Y., Yanowsky-Escatell, G., Pacheco-Moisés, P., Andrade-Sierra, J., Campos-Bayardo, I., Román-Rojas, D., García-Sánchez, A., Miranda-Díaz, G. Oxidation State in Peritoneal Dialysis in Patients with Type 2 Diabetes Mellitus. *Int J Mol Sci.* 2023; Jan 31;24(3):2669. doi: 10.3390/ijms24032669.

- Pereira, J., Eriksson, W. Emerging Role of SGLT-2 Inhibitors for the Treatment of Obesity. *Drugs*. 2019 Feb;79(3):219-230. doi: 10.1007/s40265-019-1057-0.
- Perkovic, V., Jardine, J., Neal, B. Canagliflozin and renal outcomes in type 2 diabetes and nephropathy. *New England J Med*. 2019;380(24):2295-2306.
- Perrone, A., Giovino, A., Benny, J., Martinelli, F. Advanced Glycation End Products (AGEs): Biochemistry, signaling, analytical methods, and epigenetic effects. *Oxidative Med. Cell. Longev*. 2020, 2020, 3818196.
- Petrovic, S., Arsic, A., Ristic-Medic, D., Cvetkovic, Z., Vucic, V. Lipid peroxidation and antioxidant supplementation in neurodegenerative diseases: A review of human studies. *Antioxidants* 2020, 9, 1128.
- Pheiffer, C., Pillay-van Wyk, V., Turawa, E., Levitt, N., Kengne, P., Bradshaw, D. Prevalence of Type 2 Diabetes in South Africa: A Systematic Review and Meta-Analysis. *Int. J. Environ. Res. Public Health*. 2021, 18, 5868. <https://doi.org/10.3390/ijerph18115868>
- Pheiffer, C., Victoria, P., Jané D. Joubert, Naomi S Levitt, Mweete Debra Nglazi and Debbie Bradshaw. “The prevalence of type 2 diabetes in South Africa: a systematic review protocol.” *BMJ Open*. 2018: n. pag.
- Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. Oxidative Stress: Harms and Benefits for Human Health. *Oxid Med Cell Longev* 2017, 8416763. <https://doi.org/10.1155/2017/8416763>
- Plaza, L., Sánchez-Moreno, C., de Pascual-Teresa, S., de Ancos, B., Cano, P. Fatty Acids, Sterols, and Antioxidant Activity in Minimally Processed Avocados during Refrigerated Storage. *J. Agric. Food Chem*. 2009, 57, 3204–3209
- Pourghassem-Gargari, Bahram, Somayeh Abedini, Hossein Babaei, Akbar Aliasgarzadeh and P Pourabdollahi. “Effect of supplementation with grape seed (*Vitis vinifera*) extract on antioxidant status and lipid peroxidation in patient with type II diabetes.” *J. Med. Plant Res*. 2011; 2029-2034.
- Prasad, N., Chew, Y., Khoo, E., Kong, W., Azlan, A.; Ismail, A. Antioxidant capacities of peel, pulp, and seed fractions of *Canarium odontophyllum* Miq. fruit. *J. Biomed. Biotechnol*. 2010, 2010, 871379. <https://doi.org/10.1155/2010/871379>
- Qi S.J, H. Huang, J. Y. Huang, Q. Y. Wang and Q. Y. Wei, Lychee (*Litchi chinensis* Sonn.) seed water extract as a potential antioxidant and anti-obese natural additive in meat products, *Food Control*, 2015, 50, 195–201.

- Queiroz, R., Abreu, C., Rocha, A., Sousa, V., Fráguas, M., Braga, A., César, P. Lychee (*Litchi chinensis* Sonn.) peel flour: Effects on hepatoprotection and dyslipidemia induced by a hypercholesterolemic diet. *An Acad Bras Cienc.* 2018, 90(1), 267–281
- Rafiq, S., Kaul, R., Sofi, A., Bashir, N. Nazir, F., Nayik, A. Citrus peel as a source of functional ingredient: A review. *J. Saudi Soc. Agric. Sci.* 2018, 17, 351–358.
- Rahman, N., Sabang, M., Abdullah, R., Bohari, B. Antioxidant properties of the methanolic extract of avocado fruit peel (*Persea americana* Mill.) from Indonesia. *J. Adv. Pharm. Technol. Res.* 2022, 13, 166–170.
- Rai, K. "Glycaemic evaluation of Psidium guajava in rats." *Indian J Med Res* 126. 2007: 224-227
- Rai, K., Asthana, P., Jaiswal, S. Biotechnological advances in guava (*Psidium guajava* L.): recent developments and prospects for further research. *Trees* 24, 1–12 2010. <https://doi.org/10.1007/s00468-009-0384-2>
- Rains, L., Jain, K. Oxidative stress, insulin signaling, and diabetes. *Free Radic. Biol. Med* 2011; 50(5):567–575. doi: 10.1016/j.freeradbiomed.2010.12.006.
- Rasouli H., Farzaei, H., Khodarahmi, R. Polyphenols and their benefits: A review. *Int. J. Food Prop.* 2017;20(2):1700-41.
- Rauf, A., Imran, M., Abu-Izneid, T., Iahtisham-Ul-Haq, Patel, S., Pan, X., Naz, S., Sanches Silva, A., Saeed, F., & Rasul Suleria, H. A. Proanthocyanidins: A comprehensive review. *Biomed Pharmacother* 2019; 116, 108999. <https://doi.org/10.1016/j.biopha.2019.108999>
- Rawshani, A., Rawshani, A., Franzén, S., Eliasson, B., Svensson, M., Miftaraj, M. Mortality and cardiovascular disease in type 1 and type 2 diabetes. *N Engl J Med* 2017; 376:1407-18
- Rayego-Mateos, S., José, M., Lucas, O., Melania, G., Cristina, G., Cristina, V., Sebastián, M., Ana Belén S., Carmen, H., Sergio, M., Carmen, G., Juan, M., and Jesus, E. "Pathogenic Pathways and Therapeutic Approaches Targeting Inflammation in Diabetic Nephropathy." *Int. J. Mol. Sci.* 21 2020; n. pag.
- Reczek, R., Chandel, S. The two faces of reactive oxygen species in cancer. *Annu. Rev. Cancer Biol.* 2017, 1, 79–98.
- Ren, S., Xu, D. D., Gao, Y., Ma, Y. T., & Gao, Q. P. Flavonoids from litchi (*Litchi chinensis* Sonn.) seeds and their inhibitory activities on α -glucosidase. *Chem res Chinese u.*,2013; 29, 682–685
- Ren, S., Xu, D., Pan, Z., Gao, Y., Jiang, Z., & Gao, Q. Two flavanone compounds from litchi (*Litchi chinensis* Sonn.) seeds, one previously unreported, and appraisal of their α -glucosidase

- inhibitory activities. *Food Chem.*, 2011; 127, 1760–1763.
<https://doi.org/10.1016/j.foodchem.2011.02.054>
- Rena, G., Hardie, D. G., & Pearson, E. R. The mechanisms of action of metformin. *Diabetologia*, 2017, 60(9), 1577–1585.
- Rifna, E.J., Misra, N., Dwivedi, M. Recent advances in extraction technologies for recovery of bioactive compounds derived from fruit and vegetable waste peels: A review. *Crit. Rev. Food Sci. Nutr.* 2021, 1–34.
- Rinaldi, C., Donato, L., Alibrandi, S., Scimone, C., D'Angelo, R., Sidoti, A. Oxidative stress and neurovascular unit. *Life* 2021, 11, 767.
- Robinson, S., Boye, S., Mody, R., Strizek, A., Konig, M., Malik, E., Kennedy-Martin T. Real-World Effectiveness of Dulaglutide in Patients with Type 2 Diabetes Mellitus: A Literature Review. *Diabetes Ther.* 2020 Jul;11(7):1437-1466. doi: 10.1007/s13300-020-00839-5.
- Röder, V., Wu, B., Liu, Y., & Han, W. Pancreatic regulation of glucose homeostasis. *Exp. Mol. Med.*, 2016 48(3), e219. <https://doi.org/10.1038/emm.2016.6>
- Rodrigues, M., Rebocho A, Ahmadpour E, Nissapatorn V, de Lourdes Pereira M. Type 1 Diabetes Mellitus: A Review on Advances and Challenges in Creating Insulin Producing Devices. *Micromachines* 2023 Jan 6;14(1):151. doi: 10.3390/mi14010151.
- Rodriguez-Carpena, G., Morcuende, D., Andrade, J., Kylli, P., Estevez, M. Avocado (*Persea americana* Mill.) phenolics, in vitro antioxidant and antimicrobial activities, and inhibition of lipid and protein oxidation in porcine patties. *J. Agric. Food Chem.* 2011, 59, 5625–5635.
- Rodríguez-Martínez, B., Ferreira-Santos, P., Gullón, B., Teixeira, J., Botelho, C., Yáñez, R. Exploiting the potential of bioactive molecules extracted by ultrasounds from avocado peels—Food and nutraceutical applications. *Antioxidants* 2021, 10, 1475.
- Rojas-García, A., Villegas-Aguilar, C., García-Villegas, A., Cádiz-Gurrea, L., Fernández-Ochoa, Á., Fernández-Moreno, P., Arráez-Román, D., Segura-Carretero, A. Characterization and biological analysis of avocado seed and peel extracts for the development of new therapeutical strategies. *Biol. Life Sci. Forum* 2022, 18, 9.
- Rong, S., Hu, X., Zhao, S., Zhao, Y., Xiao, X., Bao, W., Liu, L. Procyanidins extracted from the litchi pericarp ameliorate atherosclerosis in ApoE knockout mice: Their effects on nitric oxide bioavailability and oxidative stress. *Food Funct.* 2017, 8(11), 4210–4216.
<https://doi.org/10.1039/C7FO00747G>
- Rong, S., Zhao, S., Kai, X., Zhang, L., Zhao, Y., Xiao, X., Bao, W., & Liu, L. Procyanidins extracted from the litchi pericarp attenuate atherosclerosis and hyperlipidemia associated with

- consumption of a high-fat diet in apolipoprotein-E knockout mice. *Biomed. Pharmacother.*, 2018 97, 1639–1644. <https://doi.org/10.1016/j.biopha.2017.10.139>
- Rudich, A., Tirosh, R., Potashnik, M. Khamaisi, N. Bashan, Lipoic acid protects against oxidative stress-induced impairment in insulin stimulation of protein kinase B and glucose transport in 3T3-L1 adipocytes, *Diabetologia* 42(8) 1999; 949-57.
- Rutledge, A., Kaufman, A., Dezfulian, C., Elmer, J. Metformin protects against cardiac and renal damage in diabetic cardiac arrest patients. *Resuscitation* 2022, 174, 42–46.
- Ryan, G., Briscoe, A., Jobe, L. Review of pramlintide as adjunctive therapy in treatment of type 1 and type 2 diabetes. *Drug Des Devel Ther.* 2009; 2:203-214.
- Sagar, N.A., Pareek, S., Sharma, S., Yahia, M., Lobo, G. Fruit and Vegetable Waste: Bioactive Compounds, Their Extraction, and Possible Utilization. *Compr. Rev. Food Sci. Food Saf.* 2018, 17, 512–531
- Sahadew, N., Singaram, S. A diabetes profile of the eight districts in the public health sector, Eastern Cape Province, South Africa. *S Afr Med J.* 2019;109(12):957–62.
- Saini, K., Sharma, S., Khan, Y. DPP-4 inhibitors for treating T2DM - hype or hope? an analysis based on the current literature. *Front Mol Biosci.* 2023 May 23;10:1130625. doi: 10.3389/fmolb.2023.1130625.
- Saiteja, C., Gahangir, H., Ayush, G., Anupama, B., Sayantan, B., Devottam, G., Sanju, T., Smart home health monitoring system for predicting type 2 diabetes and hypertension. *J. King Saud Univ. Sci. Compu. Info. Sci.* Volume 34, Issue 3, 2022, Pages 862-870, <https://doi.org/10.1016/j.jksuci.2020.01.010>.
- Sakaguchi, K., Takeda, K., Maeda, M., Ogawa, W., Sato, T., Okada, S., Ohnishi, Y., Nakajima, H., & Kashiwagi, A. Glucose area under the curve during oral glucose tolerance test as an index of glucose intolerance. *Diabetol. Int.*, 2015 7(1), 53–58. <https://doi.org/10.1007/s13340-015-0212-4>
- Sakurai, T., Kitadate, K., Nishioka, H. Oligomerized lychee fruit-derived polyphenol attenuates cognitive impairment in senescence-accelerated mice and endoplasmic reticulum stress in neuronal cells. *BrJ Nutr* 2013; 110:1549-58.
- Salehi, B., Ata, A., Kumar, N., Sharopov, F., Ramírez-Alarcón, K., Ruiz-Ortega A, Abdulmajid Ayatollahi S, Tsouh Fokou PV, Kobarfard F, Amiruddin Zakaria Z, Iriti M, Taheri Y, Martorell M, Sureda A, Setzer WN, Durazzo A, Lucarini M, Santini A, Capasso R, Ostrander EA; Attaur-Rahman; Choudhary MI, Cho WC, Sharifi-Rad J. Antidiabetic Potential of Medicinal Plants

- and Their Active Components. *Biomolecules*. 2019; Sep 30;9(10):551. doi: 10.3390/biom9100551.
- Santana, I., Castelo-Branco, V.N.; Guimarães, B.M.; Silva, L.d.O.; Peixoto, V.O.D.S.; Cabral, L.M.C.; Freitas, S.P.; Torres, A.G. Hass avocado (*Persea americana* Mill.) oil enriched in phenolic compounds and tocopherols by expeller-pressing the unpeeled microwave dried fruit. *Food Chem*. 2019, 286, 354–361.
- Sanyaolu, A., Marinkovic, A., Prakash, S., Williams, M., Dixon, Y., Okorie, C., Orish, N., Izurieta, R. Diabetes mellitus: An overview of the types, prevalence, comorbidity, complication, genetics, economic implication, and treatment. *World J Meta-Anal* 2023; 11(5): 134-143
- Sarkar T, Nayak P., and Chakraborty, R. Litchi (*Litchi chinensis* Sonn.) Products and Processing Technologies: an Update, *Ambient Sci.*, 2018, 5, 11–16.
- Satyam, D., and Umeshkumar. “Macrovascular and Microvascular Complications in Newly Diagnosed Type 2 Diabetes Mellitus. *Indian J Public Health Res Dev* 2021; n. pag.
- Scarano, A., Laddomada, B., Blando, F., De Santis, S., Verna, G., Chieppa, M., Santino, A. The Chelating Ability of Plant Polyphenols Can Affect Iron Homeostasis and Gut Microbiota. *Antioxidants*. 2023,12(3), 630. <https://doi.org/10.3390/antiox12030630>
- Schena, P., Gesualdo, L. Pathogenetic mechanisms of diabetic nephropathy. *J. Am. Soc. Nephrol*. 2005;16,S30–S33.
- Segovia, J., Hidalgo, I., Villasante, J., Ramis, X., Almajano, P. Avocado seed: A comparative study of antioxidant content and capacity in protecting oil models from oxidation. *Molecules* 2018, 23, 2421
- Segura, N., Amarillo, M., Martinez, N., Grompone, M. Improvement in the extraction of Hass avocado virgin oil by ultrasound application. *J. Food Res*. 2018, 7, 106–113
- Selby, M., Taal W. An updated overview of diabetic nephropathy: Diagnosis, prognosis, treatment goals and latest guidelines. *Diabetes Obes Metab* 2020; 22: 3–15.
- Semeraro, F., Morescalchi, F., Cancarini, A., Russo, A., Rezzola, S., Costagliola, C. Diabetic retinopathy, a vascular and inflammatory disease: Therapeutic implications, *Diabetes & Metabolism*, Volume 45, Issue 6, 2019, Pages 517-527, ISSN 1262-3636, <https://doi.org/10.1016/j.diabet.2019.04.002>
- Sha, P., Modak, D., Sarkar, S., Roy, K., Sah, P., Ghatani, K., Bhattacharjee, S. Fruit waste: A current perspective for the sustainable production of pharmacological, nutraceutical, and bioactive resources. *Front. Microbiol*. 2023, 14, 1260071.

- Shaki, F., Shayeste, Y., Karami, M., Akbari, E., Rezaei, M., Ataee, R. The effect of epicatechin on oxidative stress and mitochondrial damage induced by homocysteine using isolated rat hippocampus mitochondria. *Pharm. Sci. Res.* 2017, 12(2), 119–127. <https://doi.org/10.4103/1735-5362.202450>
- She, H., and Mao, Z. “Regulation of myocyte enhancer factor-2 transcription factors by neurotoxins,” *Neurotoxicology*, 2011; vol. 32, no. 5, pp. 563–566.
- Sigal, J., Kenny, P., Wasserman, H., Castaneda-Sceppa, C. Physical activity/exercise and type 2 diabetes: A consensus statement from the American Diabetes Association. *Diabetes Care*. 2004; 29:1433–1438. doi: 10.2337/dc06-9910.
- Sims-Robinson, C., Kim, B., Rosko, A., How does diabetes accelerate Alzheimer disease pathology? *Nat Rev Neurol*; 2010; 6(10): 551-559
- Singla, K., Dubey, K., Garg, A., Sharma, K., Fiorino, M., Ameen, M. Natural polyphenols: chemical classification, definition of classes, subcategories, and Structures. Oxford University Press 2019.
- Sirdah, M., and Scott, R. “Genetic predisposition in type 2 diabetes: A promising approach toward a personalized management of diabetes.” *Clinical Genetics* 2020: 525 - 547.
- Şöhretoğlu, D., Renda, G., Arroo, R., Xiao, J., Sari, S. Advances in the natural α -glucosidase inhibitors. *eFood*. 2023, 4(5), e112. <https://doi.org/10.1002/efd2.112>
- Solomon, D., Chew, E., Duh, J., Sobrin, L., Sun, K., VanderBeek, L. Diabetic retinopathy: A position statement by the American diabetes association. *Diabetes Care* 40 (3), 2017; 412–418. doi:10.2337/dc16-2641
- Soumya, D., Srilatha, B Late-Stage Complications of Diabetes and Insulin Resistance. *J Diabetes Metab* 2:167.2011. doi:10.4172/2155- 6156.1000167
- Soyoye, O., Olugbenga A., Rosemary I., Babatope, K., and Anthony, A. “Diabetes and peripheral artery disease: A review.” *World J. Diabetes* 12 2021; 827 - 838.
- Spencer, G., Estevez, J., Liu, E. Pericytes, inflammation, and diabetic retinopathy. *Inflammopharmacology*. 2020;28(3):697-709
- Steinberg, F. Oxidative stress and sarcomeric proteins. *Cir. Res.* 2013, 112, 393–405.
- Stuart, A., Howell, M., Cartwright, B., McCurry, M., Lee, M., Ramsey, M., Stone, M., Insulin resistance and muscle insulin receptor substrate-1 serine hyperphosphorylation, *Physiol Rep* 2(12) 2014.
- Stumvoll, M., Goldstein, J., van Haeften, W. Type 2 diabetes: Principles of pathogenesis and therapy. *Lancet* 2005, 365, 1333–1346.

- Subramani, K., Yadav, D., Mishra, M., Pakkirisamy, U., Mathiyalagen, P., Prasad, G. Prevalence of Type 2 Diabetes and Prediabetes in the Gwalior-Chambal Region of Central India. *Int J Environ Res Public Health*. 2019; Nov 26;16(23):4708. doi: 10.3390/ijerph16234708.
- Suleria, A., Barrow, J., & Dunshea, R. Screening and characterization of phenolic compounds and their antioxidant capacity in different fruit peels. *Foods*, 2020; 9(9), 1206
- Sun, C., Zhao, C., Guven, E. C., Paoli, P., Simal-Gandara, J., Ramkumar, K. M., Wang, S., Buleu, F., Pah, A., Turi, V., Damian, G., Dragan, S., Tomas, M., Khan, W., Wang, M., Delmas, D., Portillo, M. P., Dar, P., Chen, L., & Xiao, J. Dietary polyphenols as antidiabetic agents: Advances and opportunities. *Food Frontiers*, 2020; 1, 18–44. <https://doi.org/10.1002/fft2.15>
- Tajuddeen, N., Sallau, S., Musa, M., Yahaya, M., Habila, D., Ismail, M. A novel antimicrobial flavonoid from the stem bark of *Commiphora pedunculata* (Kotschy & Peyr.) Engl. *Nat. Prod. Res.* 2016, 30(10), 1109–1115. <https://doi.org/10.1080/14786419.2015.1041138>
- Talmud, J., Cooper, A., Morris, W., Dudbridge, F., Shah, T., Engmann, J., Dale, C., White, J., McLachlan, S., Zabaneh, D., Wong A, Ong KK, Gaunt T, Holmes MV, Lawlor DA, Richards M, Hardy R, Kuh D, Wareham N, Langenberg C, Ben-Shlomo Y, Wannamethee SG, Strachan MW, Kumari M, Whittaker JC, Drenos F, Kivimaki M, Hingorani AD, Price JF, Humphries SE; UCLEB Consortium. Sixty-five common genetic variants and prediction of type 2 diabetes. *Diabetes*. 2015; May;64(5):1830-40. doi: 10.2337/db14-1504.
- Tanaka, R., Mataruco, de S., Frota, G., Coelho, S., Lima, dos S., Pimentel, C., Barão, E. Lychee peel extract obtained by ultrasound-assisted extraction: bioactive compounds and functional properties. *Acta Sci. Technol.* 2024, 46(1),e66447. <https://doi.org/10.4025/actascitechnol.v46i1.66447>
- Taskinen, R., Packard, J., & Borén, J. Dietary Fructose and the Metabolic Syndrome. *Nutrients*, 2019. 11(9), 1987. <https://doi.org/10.3390/nu11091987>
- Taylor, R., Al-Mrabeh, A., Zhyzhneuskaya, S., Peters, C., Barnes, C., Aribisala, S., Hollingsworth, G., Mathers, C., Sattar, N., Lean, J. Remission of Human Type 2 Diabetes Requires Decrease in Liver and Pancreas Fat Content but Is Dependent upon Capacity for β Cell Recovery. *Cell Metab.* 2018; Oct 2;28(4):547-556.e3. doi: 10.1016/j.cmet.2018.07.003.
- Terao, J., Piskula, M., Yao, Q. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch. Biochem.* 1994, 308(1), 278–284. <https://doi.org/10.1006/abbi.1994.1039>
- Thakur, S., Gupta, K., Ali, V., Singh, P., Verma, M. Aldose reductase: a cause and a potential target for the treatment of diabetic complications. *Arch Pharm Res* 2021; 44:655–667.

- Tremocoldi, A., Rosalen, L., Franchin, M., Massarioli, P., Denny, C., Daiuto, R., Paschoal, R., Melo, S., Alencar, M. Exploration of avocado by-products as natural sources of bioactive compounds. *PLoS ONE* 2018, 13, e0192577.
- Tresserra-Rimbau, A., Arranz, S., Vallverdu-Queralt, A. New Insights into the Benefits of Polyphenols in Chronic Diseases. *Oxid Med Cell Longev.* 2017;2017:1432071. doi: 10.1155/2017/1432071. Epub 2017 Nov 19.
- Tshane, L., Mashele, S., Matowane, R., Bonnet, L., Makhafola, J., Noreljaleel, M., Swain, S., Sekhoacha, M., Chukwuma I. Zinc(II) mineral increased the *in vitro*, cellular and *ex vivo* antihyperglycemic and antioxidative pharmacological profile of p-hydroxybenzoic acid upon complexation. *J. Food Biochem.* 2021 45(2): e13609. <https://doi.org/10.1111/jfbc.13609>
- Vassy J, Durant N, Kabagambe E, et al. A genotype risk score predicts type 2 diabetes from young adulthood: the CARDIA study. *Diabetologia.* 2012; 55:2604-2612.
- Villa-Rodríguez, J.A., Molina-Corral, J., Ayala-Zavala, F., Olivas, I., González-Aguilar, .A. Effect of maturity stage on the content of fatty acids and antioxidant activity of ‘Hass’ avocado. *Food Res. Int.* 2011, 44, 1231–1237
- Vinayagam, R., Varadavenkatesan, T. & Selvaraj, R. Evaluation of the Anticoagulant and Catalytic Activities of the *Bridelia retusa* Fruit Extract-Functionalized Silver Nanoparticles. *J Clust Sci* 28, 2919–2932. 2017. <https://doi.org/10.1007/s10876-017-1270-5>
- Vinha, F., Moreira, J., Barreira, V. Physicochemical parameters, phytochemical composition and antioxidant activity of the algarvian avocado (*Persea americana* Mill.). *J. Agric. Sci.* 2013, 5, 100
- Vita Di, S., Giuseppe, A., David B., Serena, I., Roberto M., & Riccardo, B. Quantitative evaluation of the phenolic profile in fruits of six avocado (*Persea americana*) cultivars by ultra-high-performance liquid chromatography heated electrospray-mass spectrometry, *Int. J. Food Prop.* 2017; 20:6, 1302-1312, DOI: 10.1080/10942912.2016.1208225
- Vogt, L., and Joris, R.. “Introduction to Pathogenetic Mechanisms of Diabetic Nephropathy.” *Diabetic Nephropathy* 2018; n. pag.
- Volpe, O., Villar-Delfino, H., Dos Anjos P., Nogueira-Machado, A. Cellular death, reactive oxygen species (ROS) and diabetic complications. *Cell Death Dis.* 2018;9(2):119
- Vujkovic M, Keaton, J., Lynch, J., Miller, D., Zhou, J., Tcheandjieu, C., Huffman, J., Assimes, T., K. Lorenz, X. Zhu, A.T. Hilliard, R.L. Judy, J. Huang, K. M. Lee, D. Klarin, S. Pyarajan, J. Danesh, O. Discovery of 318 new risk loci for type 2 diabetes and related vascular outcomes among

- 1.4 million participants in a multi-ancestry meta-analysis, *Nat. Genet.* 52. 2020; 680–691, <https://doi.org/10.1038/s41588-020-0637-y>.
- Wada, J., Makino, H. Innate immunity in diabetes and diabetic nephropathy. *Nat. Rev. Nephrol.* 2016, 12,13.
- Wallace, C., Bailey, L., Blumberg, B., Burton-Freeman, B., Chen, O., Crowe-White, M., Drewnowski, A., Hooshmand, S., Johnson, E., Lewis, R., Fruits, vegetables, and health: A comprehensive narrative, umbrella review of the science and recommendations for enhanced public policy to improve intake. *Crit. Rev. Food Sci. Nutr.* 2020, 60, 2174–2211.
- Wang, D., Tian, L., Lv, H., Pang, Z., Li, D., Yao, Z., Wang, S. Chlorogenic acid prevents acute myocardial infarction in rats by reducing inflammatory damage and oxidative stress. *Biomed. Pharm.* 2020, 132, 110773.
- Wang, H., Cao, Z. Anti-inflammatory Effects of (-)-Epicatechin in Lipopolysaccharide-Stimulated Raw 264.7 Macrophages. *Trop. J. Med. Res.* 2014, 13(9), 1415–1419. <http://dx.doi.org/10.4314/tjpr.v13i9.6>
- Wang, L., Bordi, L., Fleming, A., Hill, M., Kris-Etherton, M. Effect of a moderate fat diet with and without avocados on lipoprotein particle number, size and subclasses in overweight and obese adults: A randomized, controlled trial. *J. Am. Heart Assoc.* 2015, 4, e001355
- Wang, L., Pan, X., Jiang, L., Chu, Y., Gao, S., Jiang, X., Zhang, Y., Chen, Y., Luo, S., Peng, C. The biological activity mechanism of chlorogenic acid and its applications in food industry: A review. *Front. Nutr.* 2022, 9, 943911.
- Wang, M., Zheng, Y., Khuong, T., Lovatt, J. Effect of harvest date on the nutritional quality and antioxidant capacity in ‘Hass’ avocado during storage. *Food Chem.* 2012, 135, 694–698
- Wasserman, H. Insulin, Muscle Glucose Uptake, and Hexokinase: Revisiting the Road Not Taken. *Physiol*, 2022. 37(3), 115–127. <https://doi.org/10.1152/physiol.00034.2021>
- Waugh, N., Cummins, E., Royle, P., Clar, C., Marien, M., Richter B. Newer agents for blood glucose control in type 2 diabetes: systematic review and economic evaluation. *Health Technol Assess* 2010;14(36)
- Wei, L., Sun X, Fan C, Li, R, Zhou S and Yu, H. The pathophysiological mechanisms underlying diabetic retinopathy. *Front. Cell Dev. Biol.* 2022 10:963615. doi: 10.3389/fcell.2022.963615
- Weinberg Sibony, Roni, Omri Segev, Saar Dor and Itamar Raz. “Drug Therapies for Diabetes.” *Int. J. Mol. Sci.* 24 2023; n. pag.

- Wen, K., Fang, X., Yang, J., Yao, Y., Nandakumar, K., Salem, M., and Cheng, K. Recent Research on Flavonoids and their Biomedical Applications, *Curr. Med. Chem.*, 2021, 28, 1042–1066
- Werfalli, M., Kassanje, R., Kalula, S., Kowal, P., Phaswana-Mafuya, N., Levitt, S. Diabetes in South African older adults: prevalence and impact on quality of life and functional disability - as assessed using SAGE Wave 1 data. *Glob Health Action*. 2018;11(1):1449924. doi: 10.1080/16549716.2018.1449924.
- Whitehead, M., Wickremasinghe, S., Osborne, A., Van Wijngaarden, P., and Martin, K. R. Diabetic retinopathy: A complex pathophysiology requiring novel therapeutic strategies. *Expert Opin. Biol. Ther.* 18 (12), 2018; 1257–1270. doi:10. 1080/14712598.2018.1545836
- Wilson, D., & Islam, S. Fructose-fed streptozotocin-injected rat: an alternative model for type 2 diabetes. *Pharmacol. Rep.* PR, 64(1), 2012 129–139. [https://doi.org/10.1016/s1734-1140\(12\)70739-9](https://doi.org/10.1016/s1734-1140(12)70739-9)
- Wu, J., Xu, Y., Liu, X., Chen, M., Zhu, B., Wang, H., Shi, S., Qin, L., & Wang, S. Isolation and structural characterization of a non-competitive α -glucosidase inhibitory polysaccharide from the seeds of *Litchi chinensis* Sonn. *Int. J. Biol. Macromol.*; 2019 154, 1105–1115. <https://doi.org/10.1016/j.ijbiomac.2019.11.170>
- Wu, M., Mei, F., Hu, K. Diabetic retinopathy and cognitive dysfunction: a systematic review and meta-analysis. *Acta Diabetol* 59, 443–459 2022; <https://doi.org/10.1007/s00592-021-01829-0>
- Wu, X., Hu, M., Hu, X., Ding, H., Gong, D., Zhang, G. Inhibitory mechanism of epicatechin gallate on α -amylase and α -glucosidase and its combinational effect with acarbose or epigallocatechin gallate. *J. Mol. Liq.* 2019, 290(15) 111202. <https://doi.org/10.1016/j.molliq.2019.111202>
- Wu, Y., Chiu, H., Yang, J., Lin, L., Tseng, K., Chen, C. Inhibitory effects of litchi (*Litchi chinensis* Sonn.) flower-water extracts on lipase activity and diet-induced obesity. *J Funct Foods* 2013; 5:923-9.
- Xianan, Z., Xin, L., Mingshen, S., Jihong, D., Huijuan, Z., Xiongwei, L., Zhengwen Y., A comparative UPLC-Q-TOF/MS-based metabolomics approach for distinguishing peach (*Prunus persica* (L.) Batsch) fruit cultivars with varying antioxidant activity, *Int. Food Res.*, Volume 137, 2020, 109531, ISSN 0963-9969, <https://doi.org/10.1016/j.foodres.2020.109531>
- Xiang, Y., Chi, Y., Han, X., Kong, P., Liang, Z., Wang, D., Xiang, H., Xie, Q. *Litchi chinensis* seed prevents obesity and modulates the gut microbiota and mycobiota compositions in high-fat diet-induced obese zebrafish. *Food Funct.* 2022; Mar 7;13(5):2832-2845. doi: 10.1039/d1fo03991a.

- Xianjun, X., Huangdi, F., Liliang, H., Xiaohui, L., Direct Amidation of Carboxylic Acids through an Active α -Acyl Enol Ester Intermediate, *J. Org. Chem.*, Volume 83, Issue 15, 2018, Pages 7962-7969, ISSN 0022-3263, <https://doi.org/10.1021/acs.joc.8b00819>.
- Xu, L., Li, W., Chen, Z., Guo, Q., Wang, C., Santhanam, K., Chen, H. Inhibitory effect of epigallocatechin-3-O-gallate on α -glucosidase and its hypoglycemic effect via targeting PI3K/AKT signaling pathway in L6 skeletal muscle cells. *Int. J. Biol. Macromol.* 2019, 125, 605–611. <https://doi.org/10.1016/j.ijbiomac.2018.12.064>
- Xu, Y., Tang, G., Zhang, C., Wang, N., Feng, Y. Gallic Acid and Diabetes Mellitus: Its Association with Oxidative Stress. *Molecules* 2021, 26, 7115. <https://doi.org/10.3390/molecules26237115>
- Yaghootkar, H., Whitcher, B., Bell, D., Thomas, L. (Royal Devon & Exeter Hospital, Exeter; University of Westminster, London, UK; and University of Technology, Lulea, Sweden). Ethnic differences in adiposity and diabetes risk – insights from genetic studies (Review Symposium). *J Intern Med* 2020; 288: 271–283
- Yahia, E., Woolf, A., Avocado (*Persea americana* Mill.), Editor(s): Elhadi M. Yahia, In Woodhead Publishing Series in Food Science, Technology, and Nutrition, Postharvest Biology and Technology of Tropical and Subtropical Fruits, Woodhead Publishing, 2011, Pages 125-186e, ISBN 9781845697341, <https://doi.org/10.1533/9780857092762.125>
- Yamanouchi, T. Concomitant therapy with pioglitazone and insulin for the treatment of type 2 diabetes. *Vasc Health Risk Manag.* 2010; Apr 15;6:189-97.
- Yang, K., Chan, C. Epicatechin potentiation of glucose-stimulated insulin secretion in INS-1 cells is not dependent on its antioxidant activity. *Acta Pharmacol Sin* 2018. 39, 893–902 <https://doi.org/10.1038/aps.2017.174>
- Yang, R., Tang, X., Zhou, Y., Wu, Q., Sun, Y. Effect of ethyl acetate extracts from litchi fruit on the Th1/Th2 cell balance in mice. *Mod. Food Sci. Technol.*, 2013 29(7):1467-1470.
- Yang, Z., Zhang, L., Liu, J., & Li, D. Litchi Pericarp Extract Treats Type 2 Diabetes Mellitus by Regulating Oxidative Stress, Inflammatory Response, and Energy Metabolism. *Antioxidants*. 2024, 13(4), 495. <https://doi.org/10.3390/antiox13040495>
- Yao, Y., Gao, J., Simal-Gandara, A., Farag, W., Chen, D., Yao, D., Delmas, Z., Chen, K., Liu, H. Hu, J., Xiao, X., Rong, S., Wang, Hu., and Wang, Y. Litchi (*Litchi chinensis* Sonn.): a comprehensive review of phytochemistry, medicinal properties, and product development, *Food Funct.*, 2021, 12, 9527–9548.

- Yao, Y., Liu, T., Yin, L., Man, S., Ye, S., & Ma, L. Polyphenol-rich extract from Litchi chinensis seeds alleviates hypertension-induced renal damage in rats. *J. Agric. Food Chem.*, 2021; 69(7), 2138–2148
- Yaribeygi, H., Sathyapalan, T., Atkin, L., Sahebkar A. Molecular Mechanisms Linking Oxidative Stress and Diabetes Mellitus. *Oxid Med Cell Longev.* 2020. Mar 9; 2020:8609213. doi: 10.1155/2020/8609213
- Yin, J., Deng, H., Qin, S., Tang, W., Zeng, L., Zhou, B. Comparison of repaglinide and metformin versus metformin alone for type 2 diabetes: A meta-analysis of randomized controlled trials. *Diabetes Res. Clin. Pract.* 2014;105, e10–e15.
- Yuan, P., Ma, D., Zhang, Y., Gao X, Wang J, Li, R., Liu, Z., Wang, T., Yang, Y., Yuan, G., Yu, X., Wang, S., Liu, J., Liu, X. Analysis of cardiovascular risks for erectile dysfunction in Chinese patients with type 2 diabetes mellitus lacking clinical symptoms of cardiovascular diseases. *Transl Androl Urol.* 2020 Dec;9(6):2500-2509. doi: 10.21037/tau-20-999.
- Zakir, M., Neha, A., Marvi, S., Reya, S., Yash, K., Muhammad, N., Maham, K., Fnu, S., Arslan, T., Muhammad, K., Mohsin, J., Manoj K., Giustino, V., Satesh, K., Mahima, K, and Tamam, M. “Cardiovascular Complications of Diabetes: From Microvascular to Macrovascular Pathways.” *Cureus* 15 2023; n. pag
- Zhang, S, Xu, M., Zhang, W., Liu C., and Chen, S. Natural Polyphenols in Metabolic Syndrome: Protective Mechanisms and Clinical Applications, *Int. J. Mol. Sci.*, 2021, 22, 6110.
- Zhang, T., Ouyang, H., Mei, X., Lu, B., Yu, Z., Chen, K., Wang, Z., Ji, L. Erianin alleviates diabetic retinopathy by reducing retinal inflammation initiated by microglial cells via inhibiting hyperglycemia-mediated ERK1/2-NF-κB signaling pathway. *FASEB J.* 2019, 33, 11776–11790
- Zhang, W., Welihinda, A., Mechanic, J., Ding H, Zhu L, Lu Y, Deng Z, Sheng Z, Lv B, Chen Y, Roberge JY, Seed B, Wang YX. EGT1442, a potent and selective SGLT2 inhibitor, attenuates blood glucose and HbA(1c) levels in db/db mice and prolongs the survival of stroke-prone rats. *Pharmacol Res.* 2011; Apr;63(4):284-93. doi: 10.1016/j.phrs.2011.01.001.
- Zhao, L., Wang, K., Wang, K., Zhu, J., & Hu, Z. Nutrient components, health benefits, and safety of litchi (*Litchi chinensis* Sonn.): A review. *Compr. Rev. Food Sci. Food Saf.* 2020, 19(4), 2139–2163. <https://doi.org/10.1111/1541-4337.12590>
- Zhao, M., Yang, B., Wang, J., Li, B., Jiang, Y. Identification of the major flavonoids from pericarp tissues of lychee fruit in relation to their antioxidant activities. *Food Chem.* 2006, 98(3), 539–544. <https://doi.org/10.1016/j.foodchem.2005.06.0283>

- Zhao, M., Yang, B., Wang, J., Liu, Y., Yu, L., Jiang, Y. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn) pericarp. *Int. Immunopharmacol.* 2007, 7(2), 162–166. <https://doi.org/10.1016/j.intimp.2006.09.003>
- Zheng, Y., Ley, H., Hu, F., Global aetiology and epidemiology of type 2 diabetes mellitus and its complications, *Nat. Rev. Endocrinol.* 2018.88–98, <https://doi.org/10.1038/nrendo.2017.151>
- Zhou, J., Ashoori, F., Suzuki, S., Nishigaki, I., Yagi, K. Protective effect of chlorogenic acid on lipid peroxidation induced in the liver of rats by carbon tetrachloride or ^{60}Co -irradiation. *J. Clin. Biochem. Nutr.* 1993, 15, 119–125
- Zhu, J., Chen, M., Pang, Y., Li, S. Impact of lifestyle education for type 2 diabetes mellitus: Protocol for a randomized controlled trial. *J. Med.* 2021, 100(1), e24208. <https://doi.org/10.1097/MD.00000000000024208>

APPENDIX

SUPPLEMENTARY MATERIALS

Table S1: Non-polar to polar eluent system used gradient column fractionation of the EtAc extract

Solvent system	v:v:v ratio (mL)
DCM: MEOH: WATER	9:1:0.125
DCM: MEOH: WATER	8:2:0.1875
DCM: MEOH: WATER	7:3:0.281
DCM: MEOH: WATER	6:4:0.422
DCM: MEOH: WATER	5:5:0.633
MEOH: ETHYL: WATER	5:5:0.633

Table S2: Yield of fractions from gradient column chromatography of the EtAc extract

Fraction	Yield (g)
1	0.2
2	0.12
3	0.14
4	0.51
5	0.47
6	0.81
7	0.13
8	0.11

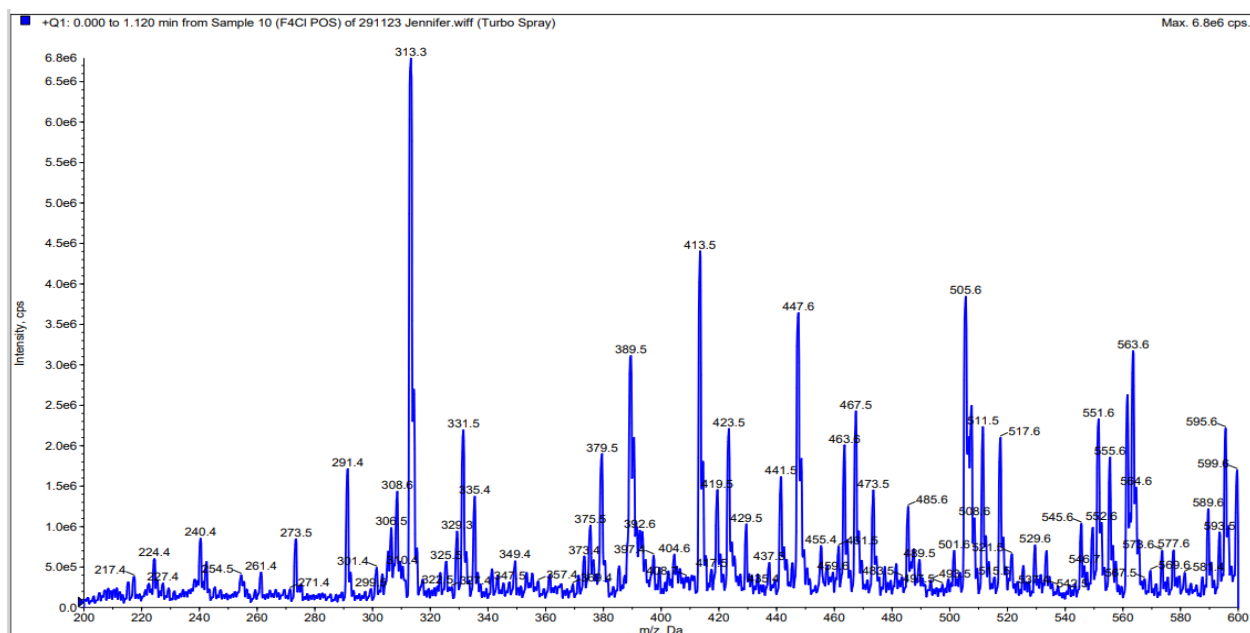


Figure S1: ESIMS (+) spectrum of the isolated compound

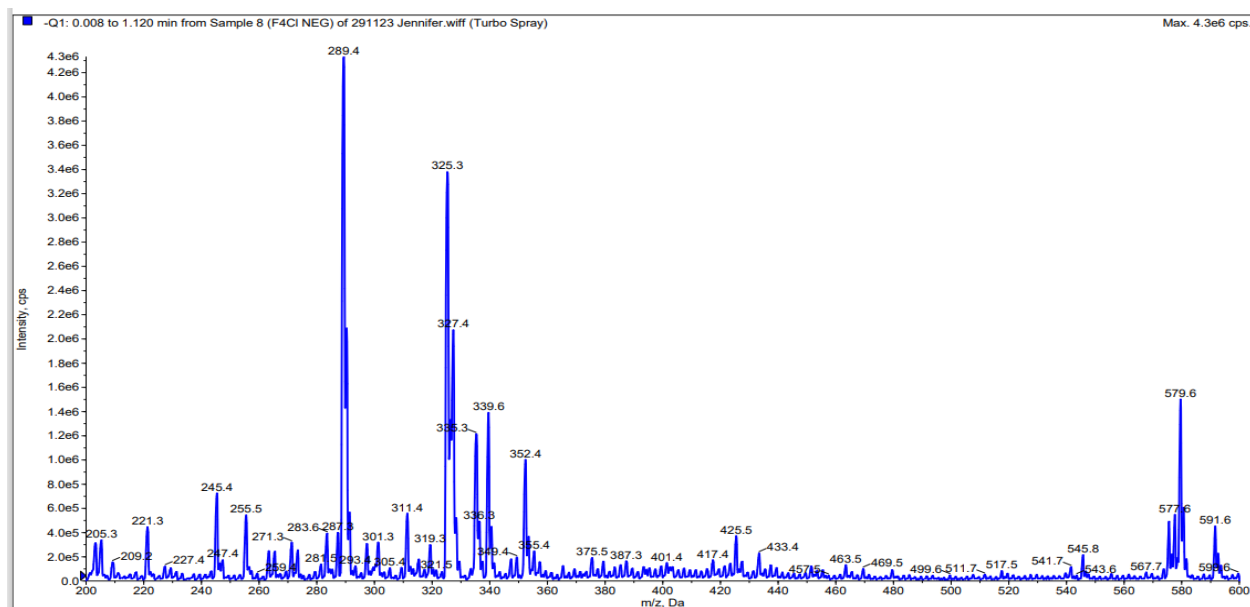


Figure S2: ESIMS (-) spectrum of the isolated compound

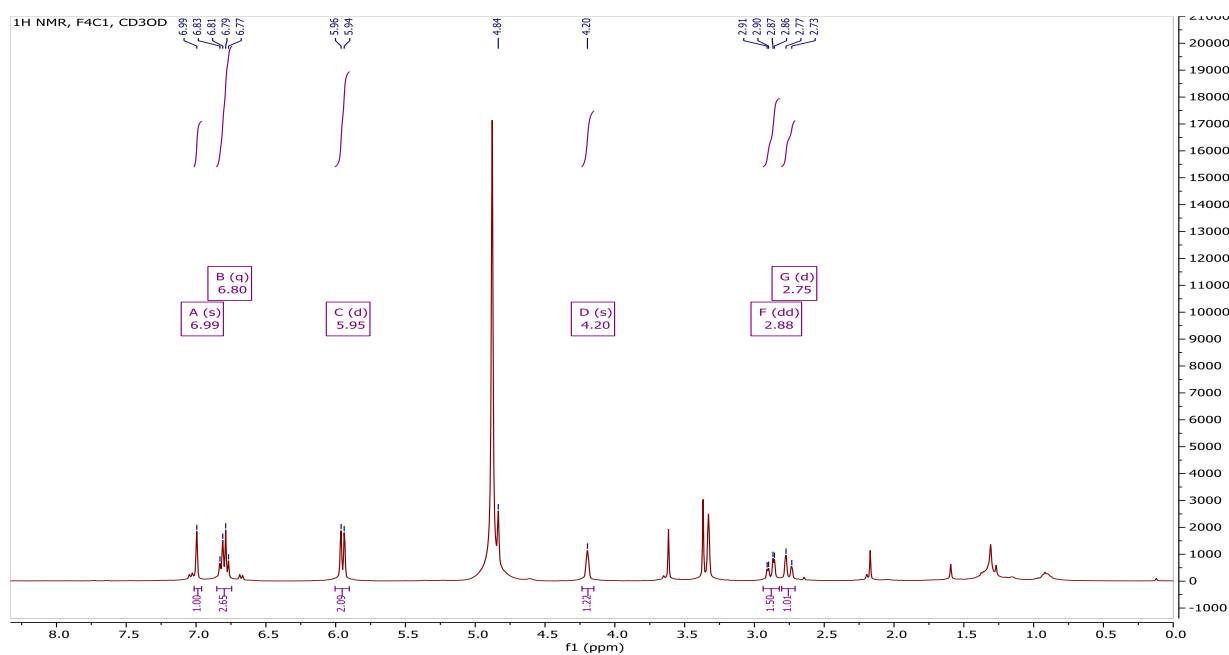


Figure S3: ^1H NMR (400 MHz, CD_3OD) spectrum of the isolated compound

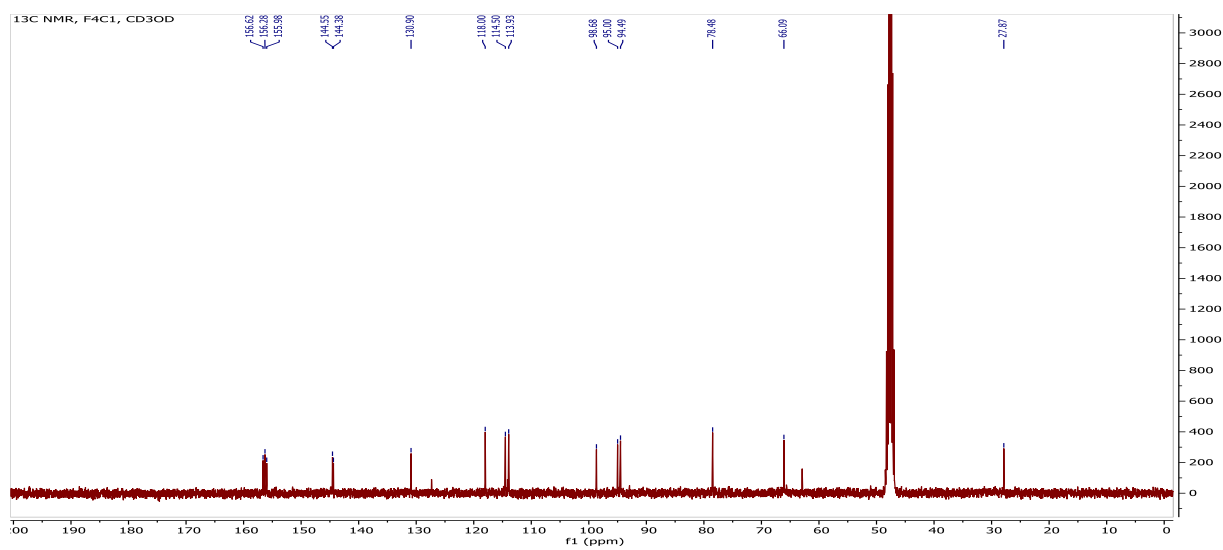


Figure S4: ^{13}C NMR (100 MHz, CD_3OD) spectrum of the isolated compound

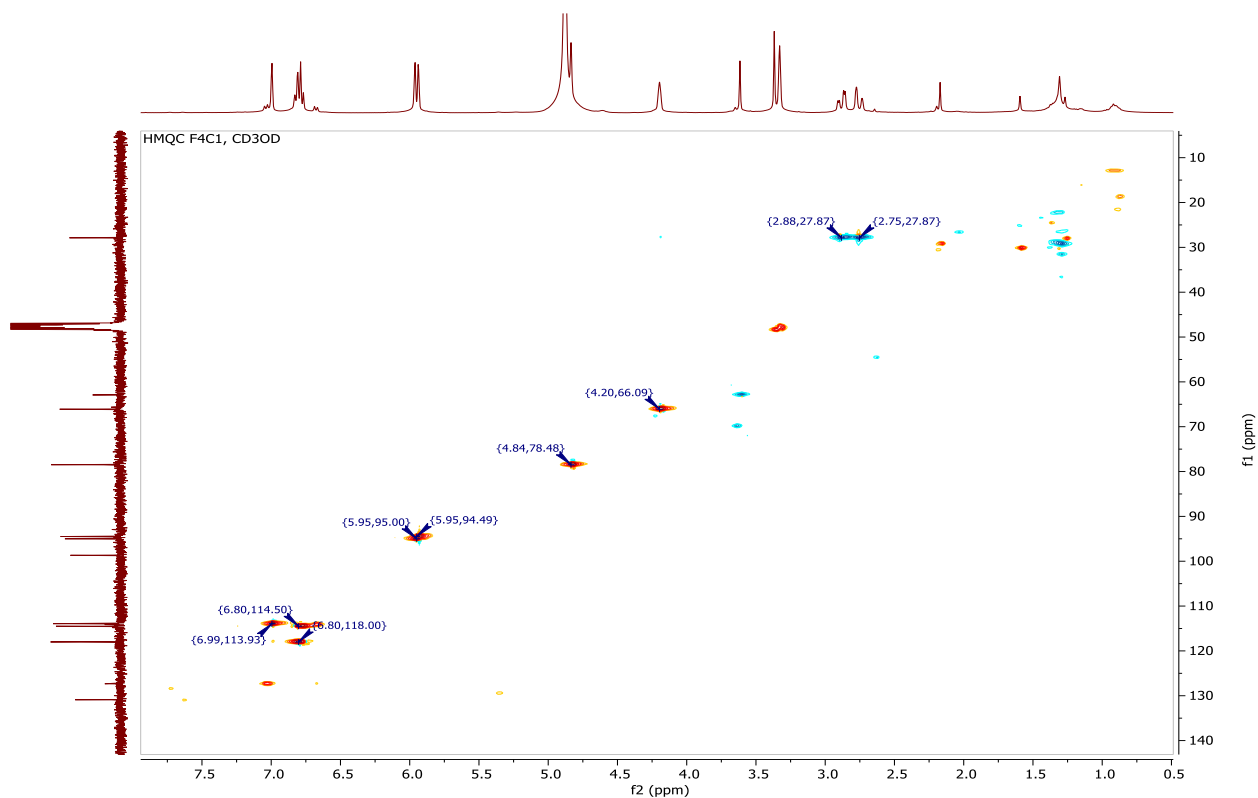


Figure S5: HSQC spectrum (CD₃OD) of the isolated compound

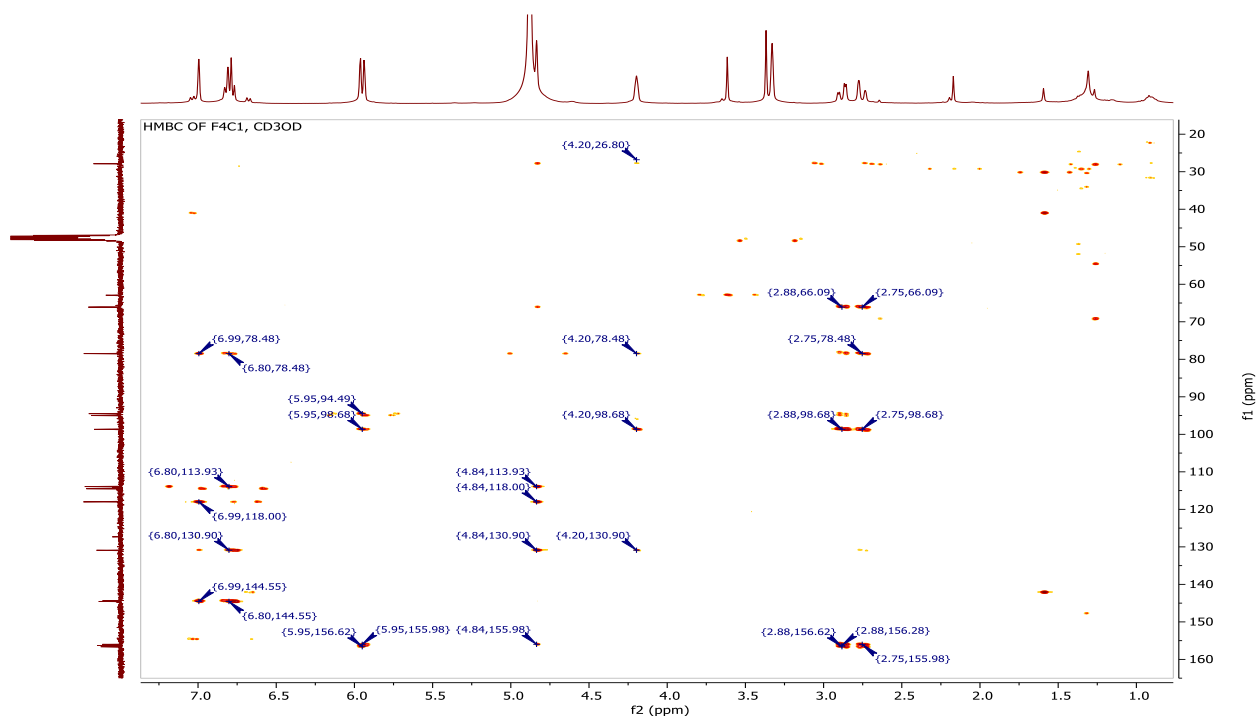


Figure S6: HMBC spectrum (CD₃OD) of the isolated compound

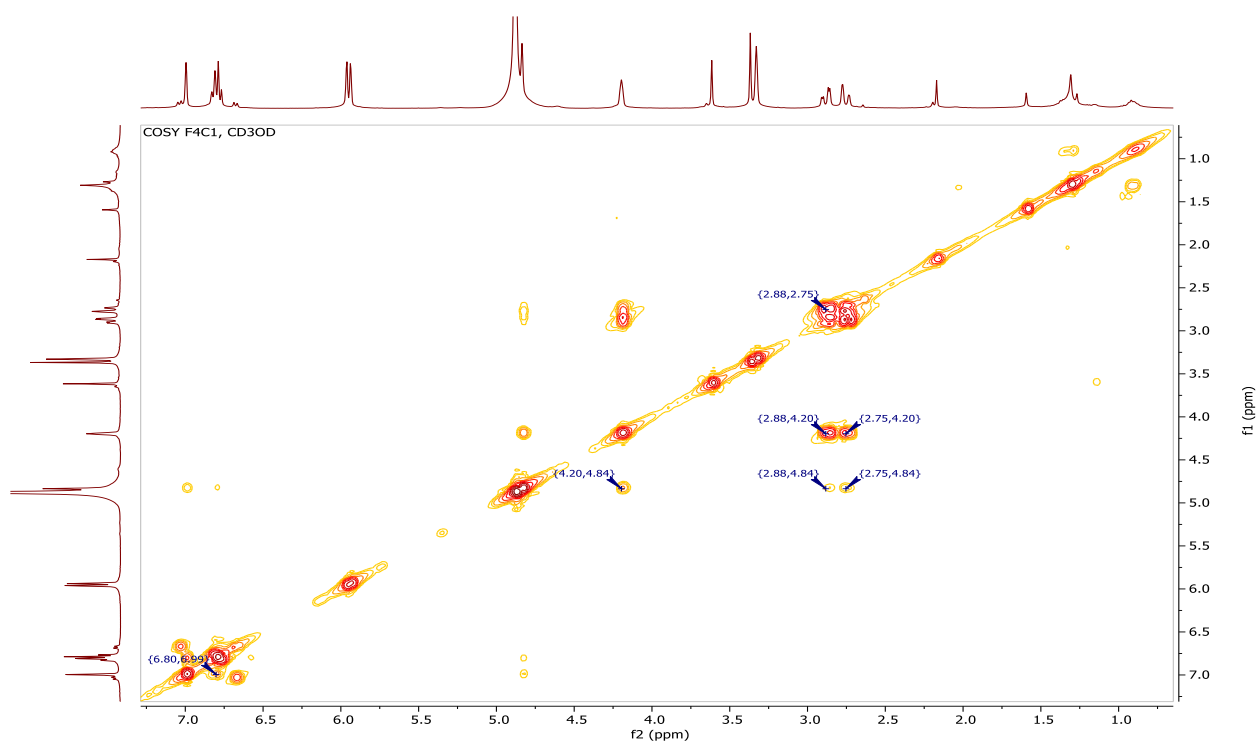


Figure S7: COSY spectrum (CD₃OD) of the isolated compound

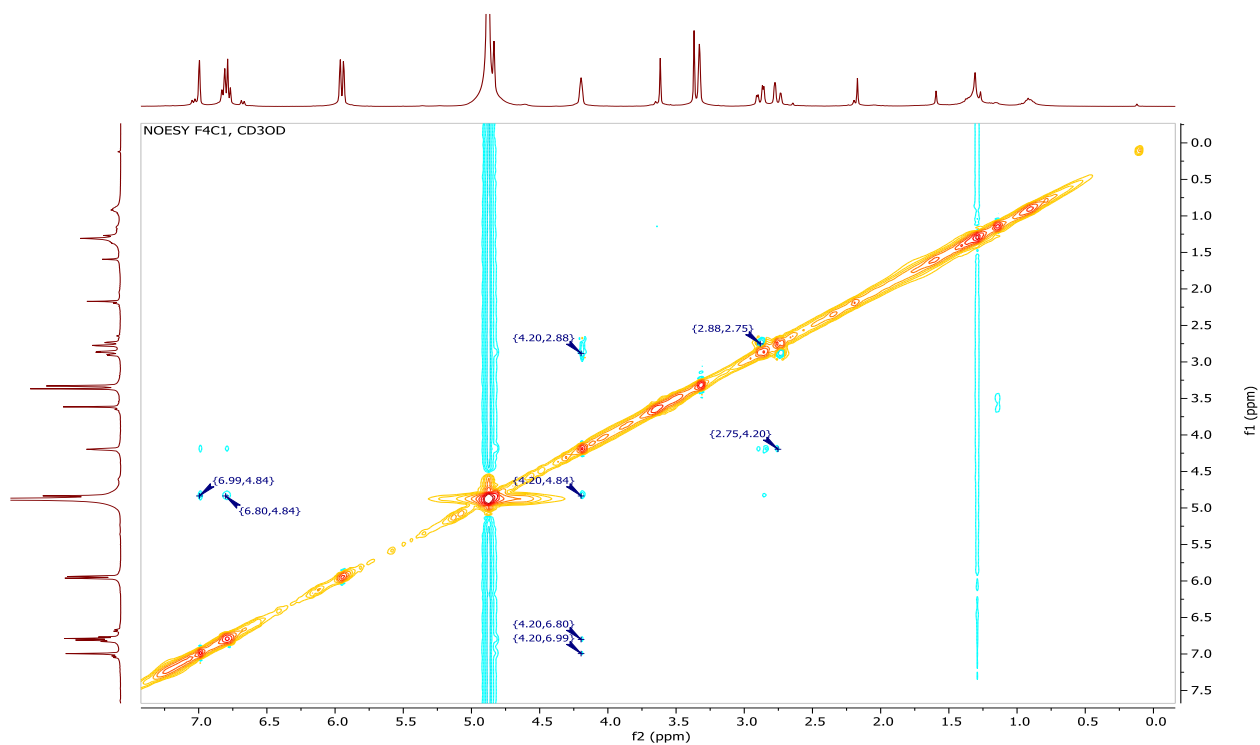


Figure S8: NOESY spectrum (CD₃OD) of the isolated compound



Article

Unravelling the Influence of Chlorogenic Acid on the Antioxidant Phytochemistry of Avocado (*Persea americana* Mill) Fruit Peel

Gloria O. Izu ¹, Emmanuel Mfotie Njoya ¹, Gaetan T. Tabakam ¹, Jennifer Namboozee ², Kgalaletso P. Otukile ², Seiso E. Tsoeu ³, Victoria O. Fasiku ⁴, Ayodeji M. Adegoke ⁴, Ochuko L. Erukainure ⁵, Samson S. Mashele ¹, Tshepiso J. Makhaola ¹, Mamello P. Sekhoacha ⁴ and Chika I. Chukwuma ^{1,*}

¹ Centre for Quality of Health and Living (CQHL), Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein 9301, South Africa; 221039621@stud.cut.ac.za (G.O.I.); enjoya@cut.ac.za (E.M.N.); tgaetan@cut.ac.za (G.T.T.); smashele@cut.ac.za (S.S.M.); jmakhaola@cut.ac.za (T.J.M.)

² Department of Chemistry, Faculty of Natural and Agricultural Sciences, University of the Free State, Bloemfontein 9301, South Africa; 2020280521@ufs4life.ac.za (J.N.); 2020930810@ufs4life.ac.za (K.P.O.)

³ Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag 3, Wits 2050, South Africa; 557872@students.wits.ac.za

⁴ Department of Pharmacology, Faculty of Health Sciences, University of the Free State, Bloemfontein 9301, South Africa; fasiku.v@ufs.ac.za (V.O.F.); adegoke.am@ufs.ac.za (A.M.A.)

⁵ Laser Research Centre, Faculty of Health Sciences, University of Johannesburg, Doornfontein 2028, South Africa

* Correspondence: chykochi@yahoo.com or cchukwuma@cut.ac.za; Tel.: +27-(0)-51-507-4028



Citation: Izu, G.O.; Mfotie Njoya, E.; Tabakam, G.T.; Namboozee, J.; Otukile, K.P.; Tsoeu, S.E.; Fasiku, V.O.; Adegoke, A.M.; Erukainure, O.L.; Mashele, S.S.; et al. Unravelling the Influence of Chlorogenic Acid on the Antioxidant Phytochemistry of Avocado (*Persea americana* Mill) Fruit Peel. *Antioxidants* **2024**, *13*, 456. <https://doi.org/10.3390/antiox13040456>

Academic Editors: Adriana Basile and Natale Badalamenti

Received: 5 March 2024

Revised: 22 March 2024

Accepted: 10 April 2024

Published: 12 April 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Oxidative stress is pivotal in the pathology of many diseases. This study investigated the antioxidant phytochemistry of avocado (*Persea americana* Mill) peel. Different solvent extracts (dichloromethane, ethyl acetate, methanol, and water) of avocado peel were subjected to total phenol and flavonoid quantification, as well as in vitro radical scavenging and ferric reducing evaluation. The methanol extract was subjected to gradient column chromatographic fractionation. Fraction 8 (eluted with hexane:chloroform:methanol volume ratio of 3:6.5:0.5, respectively) was subjected to LC-MS analysis. It was assessed for cellular inhibition of lipid peroxidation and lipopolysaccharide (LPS)-induced ROS and NO production. The DPPH radical scavenging mechanism of chlorogenic acid was investigated using Density Functional Theory (DFT). The methanol extract and fraction 8 had the highest phenol content and radical scavenging activity. Chlorogenic acid (103.5 mg/mL) and 1-O-caffeoylquinic acid (102.3 mg/mL) were the most abundant phenolics in the fraction. Fraction 8 and chlorogenic acid dose-dependently inhibited in vitro ($IC_{50} = 5.73$ and $6.17 \mu\text{g/mL}$) and cellular ($IC_{50} = 15.9$ and $9.34 \mu\text{g/mL}$) FeSO_4 -induced lipid peroxidation, as well as LPS-induced ROS ($IC_{50} = 39.6$ and $28.2 \mu\text{g/mL}$) and NO ($IC_{50} = 63.5$ and $107 \mu\text{g/mL}$) production, while modulating antioxidant enzyme activity. The fraction and chlorogenic acid were not cytotoxic. DFT analysis suggest that an electron transfer, followed by proton transfer at carbons 3'OH and 4'OH positions may be the radical scavenging mechanism of chlorogenic acid. Considering this study is bioassay-guided, it is logical to conclude that chlorogenic acid strongly influences the antioxidant capacity of avocado fruit peel.

Keywords: avocado peel; chlorogenic acid; antioxidant; chromatography

1. Introduction

Oxidative stress remains a culprit in the development and progression of many chronic diseases [1]. It is caused by a disruption in the balance between the biological production of deleterious reactive oxygen species (ROS)/reactive nitrogen species (RNS) and mitigating antioxidant actions [2,3]. ROS and RNS play important roles in redox balance and signaling, immune response, and cellular homeostasis [2]. However, most ROS and RNS are radical

species, which are very reactive [3,4]. Thus, their uncontrolled production could lead to oxidative alteration or damage of important biological molecules [1,3,4]. Oxygen, unlike other elements that exists as bi-atomic molecules, has better stability in the triplet state of electron spin (the state of unpaired electrons) than in the singlet state of electron spin (the state of paired electrons), which plays a major role in the reactivity of ROS [2].

ROS and RNS are mostly by-products of biological signaling and/or metabolism. The commonly generated ROS and RNS include hydroxyl radicals, hydrogen peroxide, nitric oxide, peroxides, peroxynitrite, singlet oxygen, and superoxides, which are common by-products of the mitochondrial electron transport, cytochrome P₄₅₀, and NADH oxidase pathways [5]. They have been reported to have detrimental effects on biological molecules, which have been implicated in oxidative stress associated diseases [1,4].

Oxidative stress is a common denominator in the pathology of many chronic diseases (diabetes, cancer, cardiovascular diseases, renal disease, mental and neurological disorders, and pulmonary diseases) and aging due to the oxidative damage caused by ROS and RNS on DNA, proteins, and membrane lipids [1]. Redox signaling is important for proper myocardial functioning and is regulated by several kinases. Oxidative stress can dysregulate this signaling, which can lead to cardiovascular disorders [1]. Oxidative stress can cause oxidative damage to contractile proteins, which may lead to contractile dysfunctions and vascular impairments [6]. ROS may induce pro-tumorigenic signaling, which may promote cancer cell proliferation and survival [7]. In diabetic state, glycation processes can lead to advanced glycation end product (AGE) production [8]. AGEs can induce oxidative stress, which induces several stress-induced transcription factors and pro-inflammatory processes. Lipid peroxidation causes oxidative modifications to biological lipids, including membrane lipids, which compromises cell membrane integrity [1]. Oxidative modifications of lipids, such as apo-lipoprotein B, may lead to formation of oxidized low-density lipoproteins, which has been implicated in the pathology of atherosclerosis [1].

The body is equipped with antioxidant mechanisms to mop deleterious ROS and mitigate the damages caused by oxidative stress [9]. These include antioxidant enzyme systems, such as catalase, superoxide dismutase, and glutathione-related enzyme systems, as well as endogenous antioxidant molecules that quench pro-oxidants or enable the functioning of antioxidant pathways [9]. Dietary or plant-derived polyphenols are known to possess antioxidant capacity, which is key for their medicinal potential in different diseases [10]. Many fruits and vegetables contain these antioxidant phenolics, which contribute to their health benefits [11]. Despite the antioxidant benefits of fruit consumption, studies have shown that the non-edible wastes of many fruits are notable sources of phenolics, which is influential in their reported medicinal properties [12–14]. For instance, the seed of avocado fruit has ethnomedicinal applications in mental disorders and obesity [15], while the peel has cosmetic applications [16].

Avocado fruit (*Persea americana* Mill) is a globally consumed fruit. The cosmetic applications and documented anticancer, antibacterial, anti-inflammatory, and anti-hypertensive potentials of the peel have been strongly linked to its antioxidant properties [16,17]. Phenolic acids, including hydroxybenzoic and hydroxycinnamic acids and flavonoids, including anthocyanins, flavanols, flavanones, and flavonols, are some of the classes of polyphenols reported in the avocado peel, which could contribute to its antioxidant effects [18–20]. The radical scavenging activity of the peel extract has been documented [18–21]. Also, the peel has been shown to exert anti-inflammatory effects in lipopolysaccharide (LPS)-treated murine macrophage cells by suppressing TNF- α expression and NO production [18].

Despite the antioxidant and anti-inflammatory potential of avocado peel, previous studies have not been able to link or correlate its principal phytoconstituents to its bioactivity through comparative experimental analysis, which will give more insight into the antioxidant phytochemistry of the fruit's peel. In the present study, computational, in vitro, and cell-based experimental models were employed to elucidate the antioxidant phytochemistry of avocado fruit peel.

2. Materials and Methods

2.1. Procurement of Fruit and Solvent Extraction of Peel

Avocado (*Persea americana* Mill) fruit (Var. Fuerte) was procured from a local fruit shop. The fruits were washed, and the peel was neatly removed. The peel was air dried and pulverized. Approximately 125 g of the pulverized peel were first defatted with hexane. Thereafter, it was sequentially extracted with dichloromethane, ethyl acetate, methanol, and water, respectively. Extraction was done at a sample-to-solvent ratio of 125 g:1.25 L, respectively, on an orbital shaker [OrbiShake, Model 262, Labotec (Pty) Ltd., Johannesburg, South Africa] set at 125 rpm. For each solvent, extraction was done three times under ambient temperature for 72 h. The organic solvent extracts were recovered by filtering, evaporating [Buchi Rotavapor® R-300, Labotec (Pty) Ltd., Johannesburg, South Africa], and drying under a fume hood. The water extract was recovered by filtering and freeze-drying (Martin Christ Alpha 1–2 LDplus Freeze Dryer, Separations, Johannesburg, South Africa). Tannins were removed from the methanol extract via solid-phase extraction in a polyamide column. All extracts were stored at -20°C .

2.2. Preliminary Phenolic Content and In Vitro Antioxidant Activity Measurements

Total phenol and flavonoid content and Fe^{3+} reducing antioxidant activity (FRAP), as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and NO radical scavenging activities were measured in the extracts using methods reported in previous publications. For total phenol and flavonoid content, FRAP, and NO radical scavenging activity, the methods reported by Chukwuma et al. [12] were used. For DPPH and ABTS radical scavenging activities, the methods reported by [22] were used. For the antioxidant assays, ascorbic acid and Trolox were used as the positive controls. For all the above-mentioned assays, the extracts and the positive controls were tested at a concentration of $45\text{ }\mu\text{g/mL}$.

2.3. Fractionation of the Methanol Extract

About 4.1 g of the methanol extract (without tannin) was subjected to a gradient column (silica gel) fractionation. Thin layer chromatography (TLC) was first used to determine the appropriate solvent system for the gradient column fractionation. The mobile phase started with a solvent system volume ratio of 8:1.5:0.5 (hexane, chloroform, and methanol, respectively) and ended with a volume ratio of 0:9:1 (hexane, chloroform, and methanol, respectively). Collected fractions with fairly similar TLC profiles were pooled together into nine different groups labelled as fractions 2 to 10. The fractions were recovered by air drying.

2.4. Antioxidant Evaluation of Fractions

Total phenol and flavonoid content, FRAP, and DPPH and ABTS radical scavenging activities were measured for all the 9 fractions and compared to positive controls (ascorbic acid and Trolox). For all the above-mentioned assays, the fractions and positive controls were tested at a concentration of $25\text{ }\mu\text{g/mL}$.

Fraction 8 (a pool of fractions with similar TLC profile), obtained from a mobile phase solvent system volume ratio of 3:6.5:0.5 (hexane, chloroform, and methanol, respectively), had the highest total phenol content and the most potent FRAP and radical scavenging activity compared to the other fractions. Thus, fraction 8 was subjected to LC-MS analysis.

2.5. Liquid Chromatography—Mass Spectroscopic (LC-MS) Quantification

To perform the LC-MS protocol, the following were used: A Waters Synapt G2 (Waters Corporation, Milford, MA, USA), ESI probe, and ESI Pos, at a 15 V cone voltage. The operation of liquid chromatography was done using an Acquity binary solvent manager. The chromatographic column used was a HSS T3 (Waters Corporation, Milford, MA, USA). It had a dimension of $2.1 \times 150\text{ mm}$ and a particle size of $1.8\text{ }\mu\text{m}$. Two solvent systems (A and B) were used as the mobile phase. Solvent system A was water containing

0.1% methanoic acid, while solvent system B was acetonitrile containing 0.1% methanoic acid. A 0.25 mL/min flow rate was applied for the gradient chromatographic separation at 30 °C. The gradient condition followed the following sequence: 100% (A):0% (B), 10 min; 72% (A):28% (B), 21 min; 60% (A):40% (B), 50 s; 0% (A):100% (B), 2 min. The fraction was dissolved in a mixture of acetonitrile and water (1:1 *v/v*), passed through a 0.22 µm filter, and injected at a volume of 20 µL. The signals of the separated compounds were recorded at 254 nm. Using the accurate masses, the compounds in the fraction were tentatively determined and semi-quantified (mg/L) relative to rutin standard, and according to the extracted ions.

2.6. Dose-Dependent Antioxidant Evaluation of Fraction 8 and Chlorogenic Acid

Dose-dependent *in vitro* and cell-based models were used to compare the antioxidant effect of fraction 8 with that of chlorogenic acid, which, together with 1-*O*-caffeoylquinic acid (1-CQA), were the most abundant compounds in the fraction. Commercial chlorogenic acid (5-*O*-caffeoylquinic or 5-CQA) purchased from Merck, Johannesburg, South Africa, was used for the assays.

2.6.1. Measurement of Dose-Dependent DPPH Radical Scavenging Activity

The dose-dependent DPPH radical scavenging activity of the samples and positive controls (ascorbic acid and Trolox) were measured at 3.75, 7.5, 15, 30, and 60 µg/mL concentrations.

2.6.2. Measurement of Linoleic Acid Peroxidation Inhibition

The ability of fraction 8 and chlorogenic acid to inhibit FeSO₄-induced (FS) linoleic acid peroxidation was performed. The detailed protocol for this assay has recently been reported in our publication [23]. In summary, linoleic acid (50 mM) peroxidation was induced using FeSO₄·7H₂O (FS) (2 mM in assay volume) after pre-treatment with the different concentrations (5, 10, 20, 40, and 80 µg/mL in assay volume) of the samples and ascorbic acid (positive control). The thiobarbituric acid (TBA) method was used to measure the level of lipid peroxidation. Absorbance readings at 532 nm were used to compute the inhibitory potential of the tested samples on linoleic acid peroxidation as follows:

$$\text{Inhibition (\%)} = \frac{(A_{\text{Negative control}} - A_{\text{Normal control}}) - (A_{\text{Test}} - A_{\text{Normal control}})}{(A_{\text{Negative control}} - A_{\text{Normal control}})} \times \frac{100}{1}$$

where “A” means “absorbance readings”; the “Normal control” denotes reaction with sample solvent or vehicle, without FS; the “Negative control” denotes reaction with sample solvent or vehicle and FS; and the “Test” denotes reaction with sample and FS.

2.6.3. Measurement of the Antioxidant Capacity in Chang Cells

The effect of the tested samples (fraction 8 and chlorogenic acid) and ascorbic acid (positive control) on lipid peroxidation and the activity of key antioxidant enzymes was measured in Chang liver cells (ATCC® CCL-13™) induced with oxidative stress. The detailed protocol for this assay has recently been reported in our publication [23]. In summary, oxidative stress was induced in cultured cells using FS (1 mM in assay volume) after pre-treatment with the different concentrations (10, 20, 40, and 80 µg/mL in assay volume) of the fraction 8, chlorogenic acid and ascorbic acid. The thiobarbituric acid (TBA) method was used to measure the level of lipid peroxidation in the cell lysate, which was extrapolated from an MDA standard plot as thiobarbituric acid reactive substances (TBARS). Also, the activities of catalase and superoxide dismutase (SOD) were measured in the lysate of cells treated with the highest concentration (80 µg/mL) of the samples using the following assay kits purchased from Merck, Johannesburg, South Africa: Catalase Assay Kit (catalog number: CAT100) and SOD Assay Kit (product number: 19160). The

inhibitory potential of the tested samples on lipid peroxidation was computed using the formula below:

$$\text{Inhibition (\%)} = \frac{(T_{\text{Negative control}} - T_{\text{Normal control}}) - (T_{\text{Test}} - T_{\text{Normal control}})}{(T_{\text{Negative control}} - T_{\text{Normal control}})} \times \frac{100}{1}$$

where “T” is the level of lipid peroxidation in the form of TBARS; the “Normal control” denotes cells treated with the sample solvent/vehicle, without FS; the “Negative control” denotes cells treated with the sample solvent/vehicle and FS; and the “Test” denotes cells treated with the sample and FS.

2.6.4. Measurement of ROS and NO Production Inhibition in RAW 264.7 Cells

The effect of the tested samples (fraction 8 and chlorogenic acid) and ascorbic acid (positive control) on ROS and NO production was measured in RAW 264.7 macrophages [American Type Culture Collection (ATCC) Rockville, MD, USA; ATCC No.: TIB-71™] treated with lipopolysaccharide (LPS) (Merck, Johannesburg, South Africa). For ROS and NO production assays, the methods reported by Marrazzo et al. [24] and Njoya et al. [25] were adopted, respectively, with slight modifications.

RAW 264.7 macrophages were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Separations, Johannesburg, South Africa) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a CO₂ incubator (EC 160, Nüve; Separations, Johannesburg, South Africa) under standard cell culture conditions (37 °C in a humidified environment with 95% air and 5% CO₂). At about 80% confluence, the cells were trypsinized with 0.25% trypsin/EDTA (Cytiva Hyclone, USA) and split at a ratio of 1:5 for further passaging. Next, the cytotoxic effect of the tested samples on the RAW 264.7 macrophages was measured at concentrations of (6.25–100 µg/mL) using standard MTT assay protocol.

For the ROS and NO production inhibition assay, confluent RAW 264.7 macrophages were seeded in black/clear-bottom 96-well plate at a density of 10,000 cells per well and incubated for 24 h. The cells were then pre-treated for 2 h with the tested samples. For the ROS production inhibition assay, the cells were treated at sample concentrations of 25, 50, and 100 µg/mL in incubation volume. For the NO production inhibition assay the cells were treated at sample concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL in incubation volume. Some wells were assigned the control and negative control groups, which contained cells treated with the sample solvents/vehicle (≤0.5% DMSO). Next, LPS (200 ng/mL in final incubation volume) was added to the wells containing the samples and the wells assigned as the negative control group, while an equivalent volume of the 0.5% DMSO was added to the wells assigned as the control group. The plates were further incubated for 24 h.

To analyze the cells for ROS production, the cells were washed with phosphate buffered saline (PBS). Next, 100 µL of fresh FBS-free culture medium, containing a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Merck, South Africa) fluorescent probe (10 µM in incubation volume), was added into each well with minimal exposure to light, and the plates were further incubated for 30 min. Thereafter, the media containing the fluorescent probe was replaced with PBS, and fluorescence was measured at 485 nm (excitation) and 535 nm (emission) using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, USA). Also, cell images were obtained using a Leica DM IL LED inverted fluorescent microscope (Separations, Johannesburg, South Africa). The inhibitory potential of the tested samples on ROS production was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(F_{\text{Negative control}} - F_{\text{Normal control}}) - (F_{\text{Test}} - F_{\text{Normal control}})}{(F_{\text{Negative control}} - F_{\text{Normal control}})} \times \frac{100}{1}$$

where “F” means “fluorescence reading”.

To analyze the cells for NO production post-LPS treatment, 100 µL of the incubation medium from each well of the 96-well microtitre plates was aliquoted into designated wells of a new 96-well transparent plate. An equal volume of Griess reagent (Merck, South Africa) was added to the wells and the plate was kept in the dark at room temperature for 15 min. Absorbance values were measured at 540 nm. The concentration of nitrite (µM) was calculated from a sodium nitrite standard linear plot ($y = 0.0029x + 0.0114$). The inhibitory potential of the tested samples on NO production was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(C_{\text{Negative control}} - C_{\text{Normal control}}) - (C_{\text{Test}} - C_{\text{Normal control}})}{(C_{\text{Negative control}} - C_{\text{Normal control}})} \times \frac{100}{1}$$

where “C” is the nitrite concentration (µM) extrapolated from the sodium nitrite standard plot.

2.7. IC₅₀ Computation

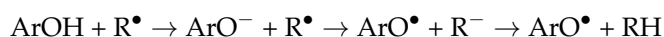
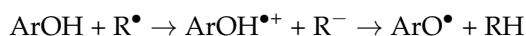
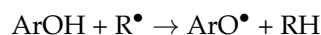
The IC₅₀ values of the tested samples were computed on GraphPad Prism 7 and/or MS excel 2016 software using the non-linear or linear plot of transformed (log 10) sample concentrations (x-axis) versus the corresponding inhibitory bioactivities.

2.8. Statistical Analysis

Experimental data was presented as mean ± standard deviation of triplicate analysis (n = 3). The statistical analysis was done on the IBM SPSS Statistics (Windows version 29.0) software using the one-way ANOVA and Tukey post-hoc multiple comparative analysis. Statistical significance was set at $p < 0.05$.

2.9. Density Functional Theory (DFT) Analysis

The hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET-PT), and sequential proton loss followed by electron transfer (SPLET) mechanisms were used to computationally study the DPPH radical scavenging activity of chlorogenic acid. The equations below sequentially denote the mechanisms for HAT, SET-PT, and SPLET, respectively.



where ArO[•], ArO[−], ArOH^{•+}, R[−], and RH denote phenolic radical, phenolic anion, phenolic cation, anion radical, and neutral molecule, respectively.

Geometry optimization of all the reaction species was performed using B3LYP together with the 6-311++g (d,p) basis set. This computational method is largely used for radical scavenging mechanisms and has been shown to accurately estimate the geometries and thermodynamic parameters of the reaction species [26]. The bond dissociation enthalpy (BDE), adiabatic ionization potential (AIP), proton dissociation enthalpy (PDE), proton affinity (PA), and electron transfer enthalpy (ETE) (in kcal/mol) of the reaction species for the capture of the DPPH radical by chlorogenic acid (5-CQA) were computed for a methanol-solvent-based reaction, which are parameters that inform the above-mentioned radical reaction mechanism.

3. Results

The methanol extract of the fruit peel had the highest ($p < 0.05$) total phenol, while the ethyl acetate extract had the highest ($p < 0.05$) total flavonoid content compared to the other extracts (Table 1). The Fe³⁺ reducing antioxidant activity of the methanol and dichloromethane extracts was significantly ($p < 0.05$) more potent than that of the other extracts and Trolox, but significantly ($p < 0.05$) less potent than that of ascorbic acid (Table 1).

The DPPH, ABTS, and NO radical scavenging activity of the methanol extract were stronger than those of the other extracts and in some instances stronger and/or comparable to those of ascorbic acid and Trolox (Table 1).

Table 1. Total phenol and flavonoid contents and in vitro Fe³⁺ reducing and radical scavenging activity of crude extract at 45 µg/mL.

Groups	Total Phenol Content (mg/g GAE)	Total Flavonoid Content (mg/g QE)	Fe ³⁺ Antioxidant Capacity (mg/g GAE)	DPPH Radical Scavenging Activity (%)	ABTS Radical Scavenging Activity (%)	NO Radical Scavenging Activity (%)
DCM	612 ± 48.2 ^b	6.94 ± 0.76 ^a	163 ± 0.61 ^b	68.1 ± 5.80 ^b	55.0 ± 5.31 ^b	52.9 ± 2.26 ^d
EtAc	142 ± 3.60 ^a	15.8 ± 2.24 ^b	31.8 ± 1.95 ^a	29.1 ± 1.26 ^a	31.8 ± 3.98 ^a	33.4 ± 4.78 ^c
MeOH	829 ± 48.6 ^c	8.17 ± 0.60 ^a	162 ± 0.42 ^b	93.9 ± 2.79 ^c	96.3 ± 3.14 ^c	62.6 ± 3.03 ^d
Water	157 ± 2.62 ^a	7.49 ± 0.19 ^a	38.7 ± 0.16 ^a	35.2 ± 1.80 ^a	40.8 ± 0.96 ^a	29.8 ± 6.51 ^{bc}
Asc	N/A	N/A	253 ± 28.1 ^c	87.5 ± 5.86 ^c	99.8 ± 0.16 ^c	11.1 ± 3.46 ^a
Trolox	N/A	N/A	57.1 ± 4.77 ^a	94.6 ± 1.25 ^c	98.1 ± 3.10 ^c	20.0 ± 2.21 ^{ab}

“ABTS”, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); “Asc”, ascorbic acid; “DCM”, dichloromethane extract; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl; “EtAc”, ethyl acetate extract; “GAE”, gallic acid equivalent; “MeOH”, methanol extract; “N/A”, not applicable; “NO”, nitric oxide; “QE”, quercetin equivalent. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Among the fractions (fractions 2 to 10) obtained from the methanol extract, fraction 8 consistently showed the highest ($p < 0.05$) total phenol content, as well as DPPH and ABTS radical scavenging and Fe³⁺ reducing antioxidant activity (Table 2). The antioxidant activity of fraction 8 was statistical comparable to that of ascorbic acid and/or Trolox (Table 2).

Table 2. Total phenol and flavonoid contents and in vitro Fe³⁺ reducing and radical scavenging activity of crude extract at 25 µg/mL.

Groups	Total Phenol Content (mg/g GAE)	Total Flavonoid Content (mg/g QE)	DPPH Radical Scavenging (%)	ABTS Radical Scavenging (%)	Fe ³⁺ Antioxidant Capacity (mg/g GAE)
F2	195 ± 1.42 ^{ab}	17.1 ± 4.00 ^{ab}	44.2 ± 1.57 ^{bc}	13.7 ± 0.21 ^b	24.8 ± 3.75 ^{ab}
F3	238 ± 12.5 ^{bc}	47.3 ± 4.10 ^{cd}	55.6 ± 1.03 ^c	40.9 ± 3.03 ^f	45.2 ± 2.52 ^b
F4	175 ± 4.21 ^a	84.45 ± 12.4 ^e	40.8 ± 1.37 ^{ab}	44.5 ± 0.03 ^f	46.4 ± 0.51 ^b
F5	236 ± 10.6 ^{bc}	8.04 ± 3.05 ^a	32.5 ± 5.6 ^a	30.1 ± 0.65 ^e	29.6 ± 0.51 ^{ab}
F6	252 ± 10.1 ^{cd}	29.3 ± 8.10 ^{bc}	43.2 ± 5.64 ^{ab}	22.5 ± 1.53 ^d	30.6 ± 6.49 ^{ab}
F7	339 ± 3.60 ^e	63.7 ± 14.9 ^{de}	68.8 ± 1.26 ^d	6.57 ± 0.01 ^a	25.6 ± 1.26 ^{ab}
F8	519 ± 48.6 ^f	39.4 ± 5.28 ^{bc}	90.8 ± 9.10 ^e	89.4 ± 4.65 ^g	96.7 ± 15.9 ^c
F9	288 ± 11.2 ^d	7.00 ± 0.40 ^a	51.9 ± 2.87 ^{bc}	15.4 ± 0.65 ^{bc}	19.0 ± 2.83 ^a
F10	179 ± 18.7 ^a	23.6 ± 7.47 ^{ab}	44.7 ± 0.33 ^b	20.6 ± 1.32 ^{cd}	44.3 ± 17.7 ^b
Asc	N/A	N/A	85.5 ± 1.32 ^e	99.4 ± 0.15 ^h	ND
Trolox	N/A	N/A	90.5 ± 2.52 ^e	99.9 ± 0.06 ^h	89.1 ± 13.0 ^c

“ABTS”, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); “Asc”, ascorbic acid; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl; “F2” to “F10”, fraction 2 to fraction 10; “GAE”, gallic acid equivalent; “N/A”, not applicable; “ND”, not determined; “QE”, quercetin equivalent. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

LC-MS analysis of fraction 8 showed the presence of several phenolic compounds, with chlorogenic acid (5-CQA) and 1-O-caffeoylquinic acid (1-CQA) being the compounds with the highest concentrations (Table 3, Figure 1, and Supplementary Material). Chlorogenic acid (5-CQA) was the compound of interest in this study.

Table 3. LC-MS data of fraction 8.

Identified Compounds	Ontology	ART (min)	AMz	Average Conc. (mg/L)
4-Hydroxy-3-prenylbenzoic acid glucoside	Phenolic glycosides	3.176	413.14206	0.1
Microperfuraneone	Butenolides	3.423	265.08621	0.5
Eudesmic acid	Gallic acid and derivatives	4.359	211.05984	0.1
Butalactin	Gamma butyrolactones	4.554	241.07141	0.1
Chlorogenic acid	Quinic acids and derivatives	4.72	353.08762	103.5
4-Hydroxybenzoic acid	Hydroxybenzoic acid derivatives	4.917	137.02409	0.1
Mycophenolic acid	Phthalides	5.014	319.12024	0.1
1-O-caffeoylquinic acid	Quinic acids and derivatives	5.029	353.0867	102.3
Caffeic acid	Hydroxycinnamic acids	5.164	179.0349	1.0
Quercetin 3-lathyroside	Flavonoid-3-O-glycosides	5.445	595.1261	21.9
Rutin	Flavonoid-3-O-glycosides	5.671	609.14667	1.0
Quercetin 3-galactoside	Flavonoid-3-O-glycosides	5.816	463.08737	30.0
Crepdiaside A	O-glycosyl compounds	5.868	421.14926	0.1
Gibberellin A17	C20-gibberellin	6.048	377.16031	0.1
Quercetin 3-[rhamnosyl-(1->2)-alpha-L-arabinopyranoside]	20-carboxylic acids	6.055	579.13745	14.2
Quercitrin	Flavonoid-3-O-glycosides	6.145	447.09045	43.4
Azelaic acid	Medium-chain fatty acids	6.41	187.09761	16.2
3-[4-[1-(4-Hydroxy-3-methoxyphenyl)-1,3-dihydroxyisopropoxy]-2-hydroxyphenyl]propyl alpha-L-rhamnopyranoside	Lignan glycosides	6.426	509.20142	0.1
Quercetin	Flavonols	6.978	301.03497	3.0
Corchorifatty acid F	Lineolic acids and derivatives	7.748	327.21799	0.1
Acrostalidic acid	Naphthopyrans	10.332	277.1442	5.4
Kopetdaghin A	Sesquiterpenoids	10.488	383.22238	0.5
Xanthoxin	Sesquiterpenoids	11.134	249.14981	16.5
etretinate	Retinoid esters	11.38	353.21255	4.2
3-Oxo-4.6-choladienoic acid	Bile acids, alcohols and derivatives	12.203	369.24011	3.4
1.1'-[1.11-Undecanediylbis(oxy)]bisbenzene	Phenol ethers	13.462	339.23151	108.8
Canrenone	Steroid lactones	14.821	339.19983	10.9

ART means "average retention time"; AMz means "average mass-to-charge ratio"; Conc. means "concentration".

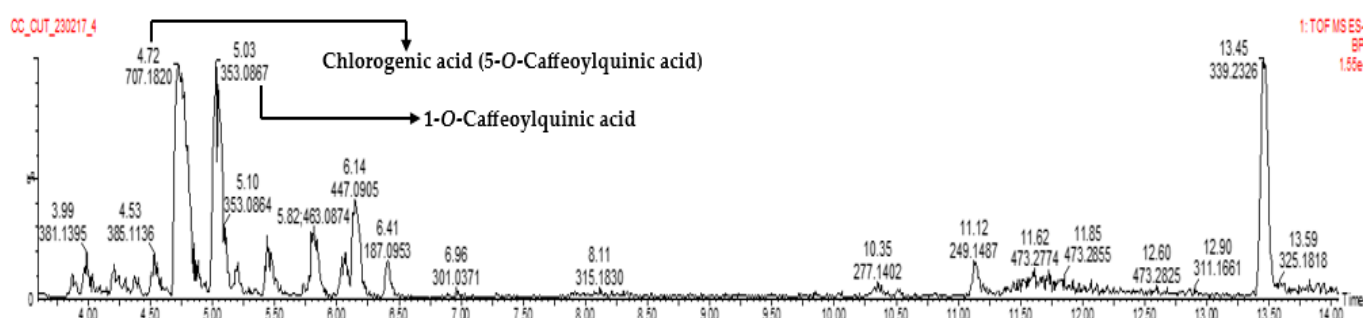


Figure 1. LC-MS chromatogram of fraction 8.

Dose-dependently, the DPPH radical scavenging activity of fraction 8 was statistically ($p > 0.05$) as potent as chlorogenic acid, ascorbic acid, and Trolox (Table 4 and Figure 2a).

Although not significantly ($p > 0.05$), the inhibitory effect of fraction 8 ($IC_{50} = 5.73 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 6.17 \mu\text{g/mL}$) on FS-induced linoleic acid peroxidation was stronger than that of ascorbic acid ($IC_{50} = 8.41 \mu\text{g/mL}$) (Table 4 and Figure 2b).

Table 4. IC_{50} values for the dose-dependent activities of the tested samples.

Assays	Fraction 8	Ascorbic Acid	Trolox	Chlorogenic Acid
	IC ₅₀ Values (μg/mL)			
DPPH radical scavenging	5.59 ± 1.75 ^{ab}	6.50 ± 2.18 ^{ab}	2.41 ± 0.46 ^b	8.84 ± 1.44 ^a
Anti-linoleic acid peroxidation	5.73 ± 1.10	8.41 ± 3.33	ND	6.17 ± 1.26
Anti-lipid peroxidation in Chang liver cells	15.9 ± 3.16 ^a	5.74 ± 0.66 ^b	ND	9.34 ± 2.40 ^b
Inhibition of ROS production in RAW 264.7 cells	39.6 ± 3.05 ^a	8.08 ± 1.83 ^c	ND	28.2 ± 2.50 ^b
Inhibition of NO production in RAW 264.7 cells	63.5 ± 4.89 ^c	203 ± 26.8 ^a	ND	107 ± 2.06 ^b

“ND” means “not determined”. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the superscript letters attached to each data represent significant differences ($p < 0.05$) between groups when there are no similar letters.

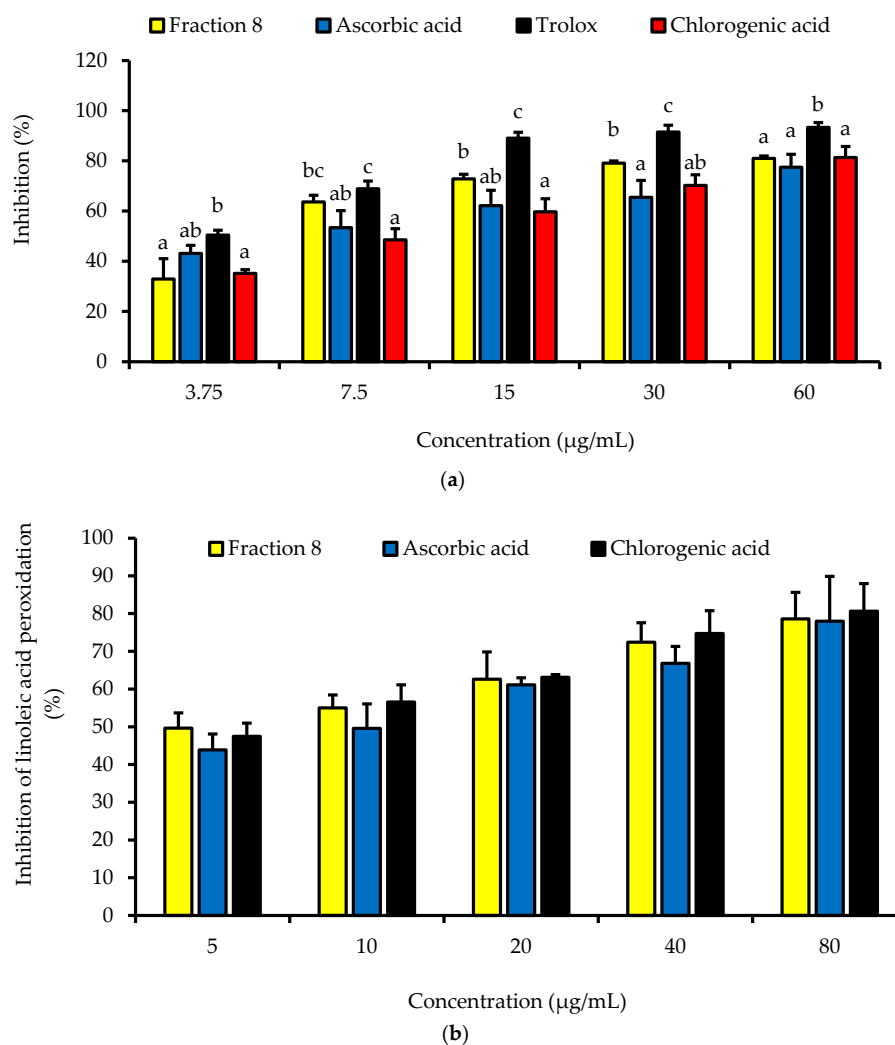


Figure 2. Dose-dependent (a) DPPH radical scavenging and (b) anti-linoleic acid peroxidative activities of fraction 8 and chlorogenic acid. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters.

In Chang cells induced with oxidative stress, the anti-lipid peroxidative of ascorbic acid ($IC_{50} = 5.74 \mu\text{g/mL}$) was significantly ($p < 0.05$) and non-significantly ($p > 0.05$) stronger than that of fraction 8 ($IC_{50} = 15.9 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 9.34 \mu\text{g/mL}$), respectively (Table 4 and Figure 3a). Nevertheless, fraction 8, chlorogenic acid, and ascorbic acid (at $80 \mu\text{g/mL}$) appreciably mitigated oxidative stress-induced depletion of catalase and SOD enzymes in the Chang cells (Figure 3b).

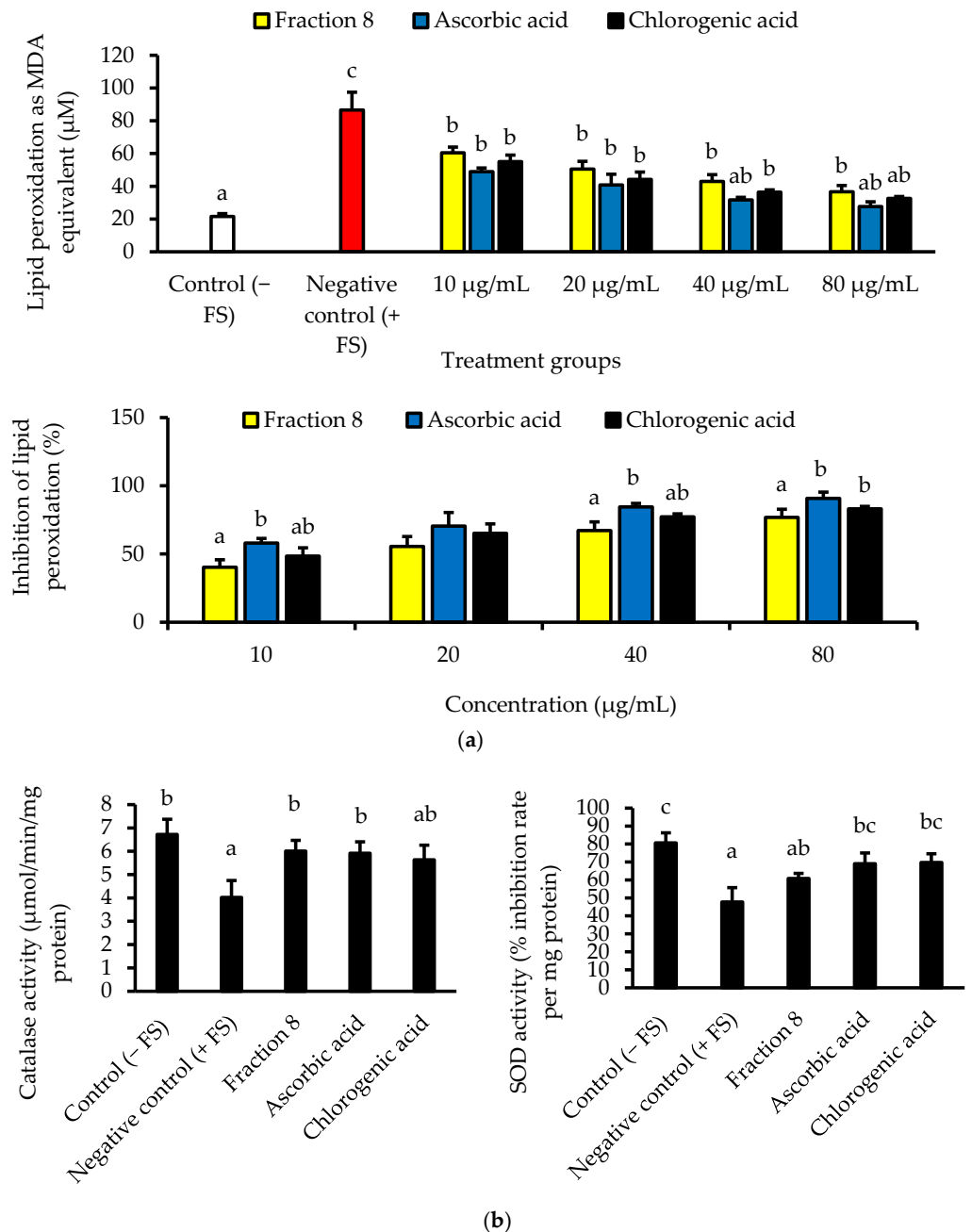


Figure 3. (a) Dose-dependent anti-lipid peroxidative and (b) antioxidant enzyme modulatory activities of fraction 8 and chlorogenic acid in FS-treated Chang cells. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done between the controls (control and negative control) and treatment groups at a given concentration or among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters. “FS” means $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

Although not as potent as ascorbic acid ($IC_{50} = 8.08 \mu\text{g/mL}$; $p < 0.05$), fraction 8 ($IC_{50} = 39.6 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 28.2 \mu\text{g/mL}$) showed promising inhibition of LPS-induced ROS production in RAW 264.7 macrophages (Table 4 and Figure 4). However, the ability of fraction 8 ($IC_{50} = 63.5 \mu\text{g/mL}$) and chlorogenic acid ($IC_{50} = 107 \mu\text{g/mL}$) to inhibit NO production in LPS-treated RAW 264.7 macrophages significantly ($p < 0.05$) outperformed that of ascorbic acid ($IC_{50} = 203 \mu\text{g/mL}$) (Table 4 and Figure 5a). Interestingly, both fraction 8 and chlorogenic acid were not toxic to the RAW 264.7 macrophages (Figure 5b).

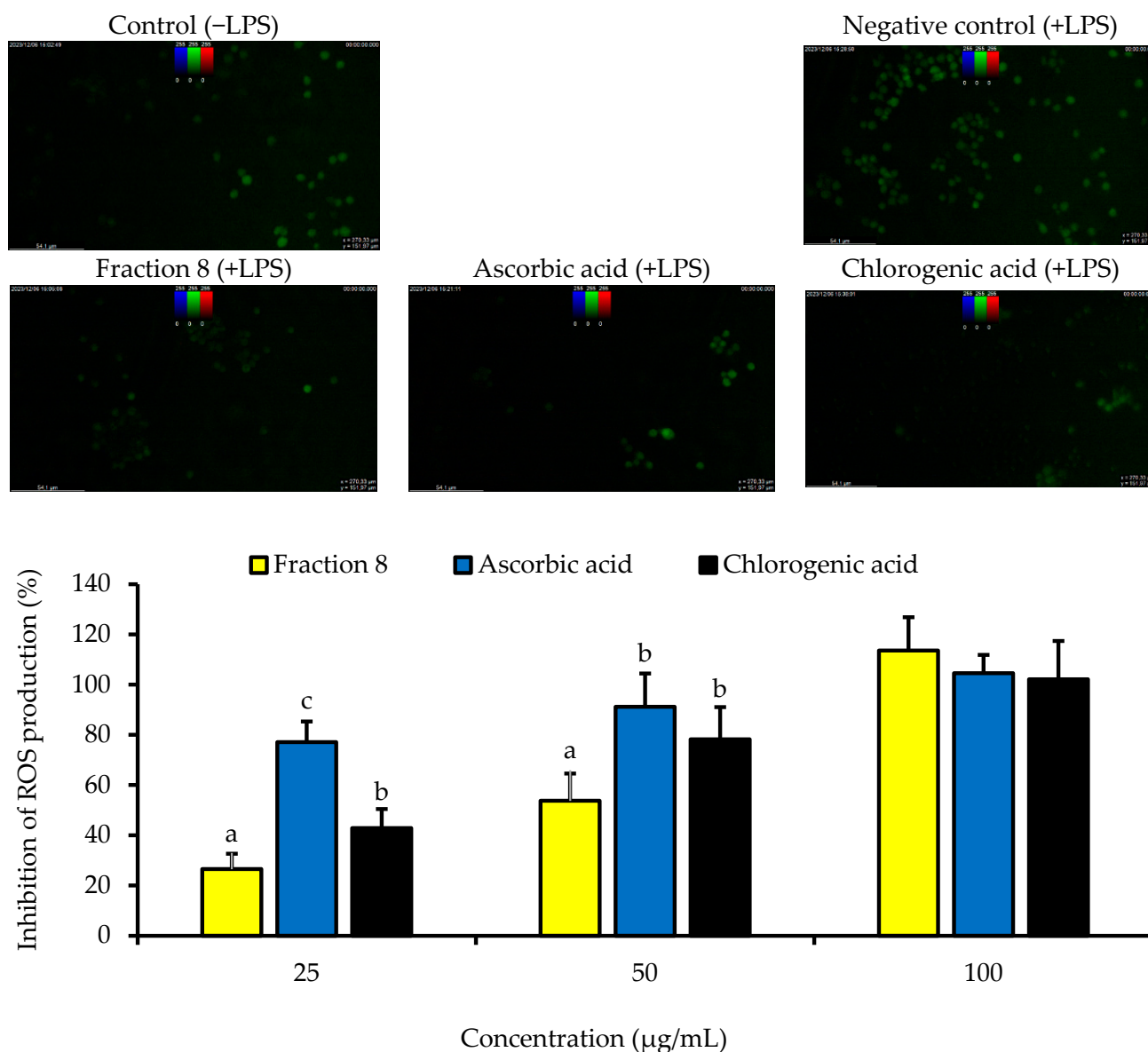
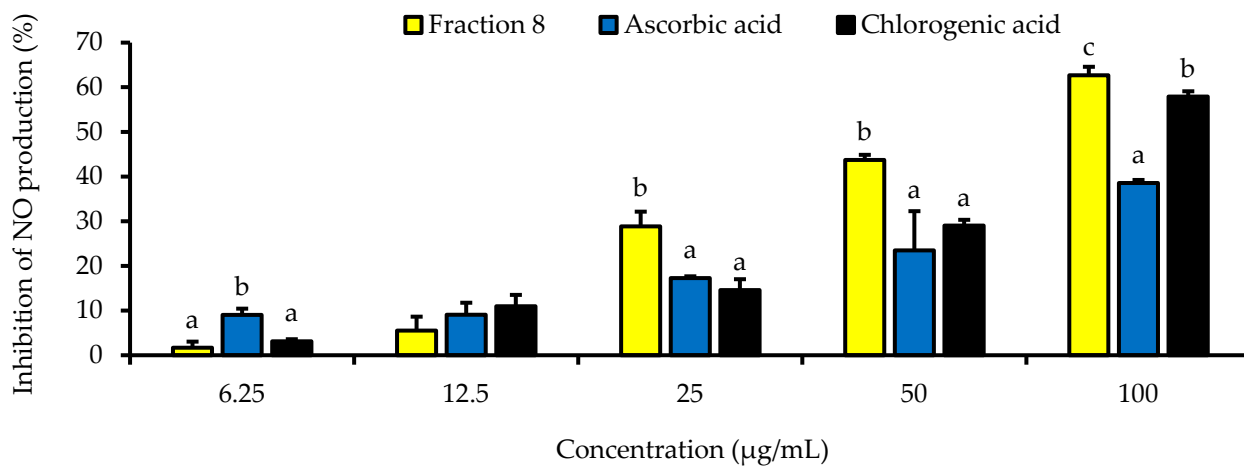
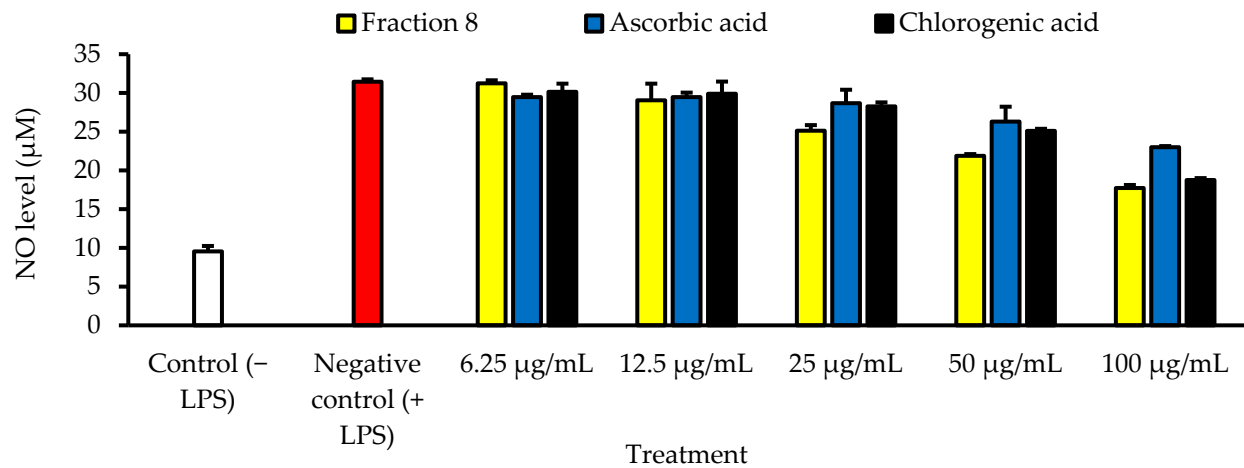
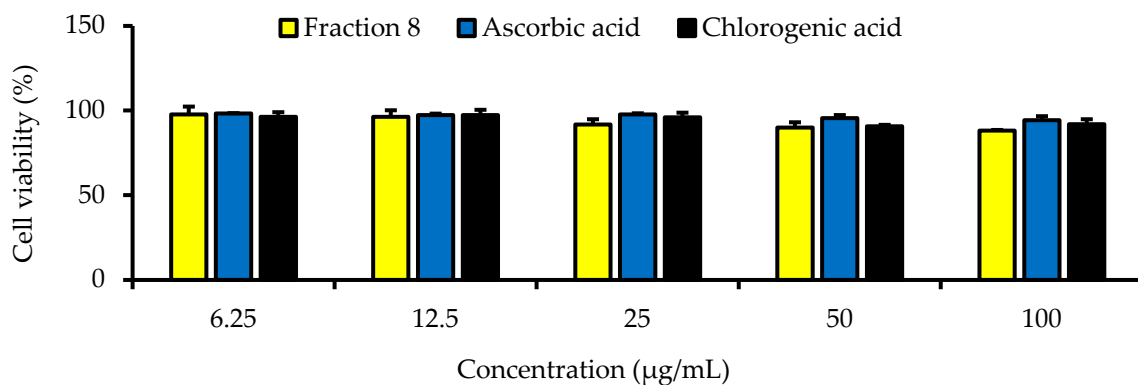


Figure 4. Dose-dependent inhibitory effect of fraction 8 and chlorogenic acid on ROS production in LPS-treated RAW 264.7 macrophages. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters. “LPS” means lipopolysaccharide.



(a)



(b)

Figure 5. Dose-dependent inhibitory effect of fraction 8 and chlorogenic acid on (a) NO production in LPS-treated RAW 264.7 macrophages and (b) the viability of RAW 264.7 macrophages. Data are presented as mean $\pm$ SD of triplicate analysis. Data are presented as mean $\pm$ SD of triplicate analysis. Statistical comparison was done between the controls (control and negative control) and treatment groups at a given concentration or among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups when there are no similar letters. “LPS” denotes “lipopolysaccharide”.

The DFT analysis data are presented in Table 5 and Figure 6. Figure 6a depicts the chemical structure of 5-CQA and the selected hydroxyl group numbering, as well as the lowest energy structural conformer of chlorogenic acid (5-CQA). Figure 6b shows the electron spin density of the optimized structures of 5-CQA radicals for HAT mechanism of DPPH radical scavenging.

Table 5. Bond dissociation enthalpy (BDE), adiabatic ionization potential (AIP), proton dissociation enthalpy (PDE), proton affinity (PA), and electron transfer enthalpy (ETE) obtained (in kcal/mol) of the reaction species for the capture of the DPPH radical via 5-CQA.

Structure *	HAT	SET-PT		SPLET	
	BDE	AIP	PDE	PA	ETE
	kcal/mol				
5-CQA-1OH	27.6	22.0	5.6	14.0	13.6
5-CQA-3OH	24.7	22.0	2.6	22.1	2.6
5-CQA-4OH	27.1	22.0	5.0	24.1	3.0
5-CQA-3'OH	2.6	22.0	−19.4	5.0	−2.4
5-CQA-4'OH	2.1	22.0	−19.9	5.3	−3.2

* The numbers 1, 3, 4, 3', and 4' denote the position of the eliminated hydrogen from 5-CQA.

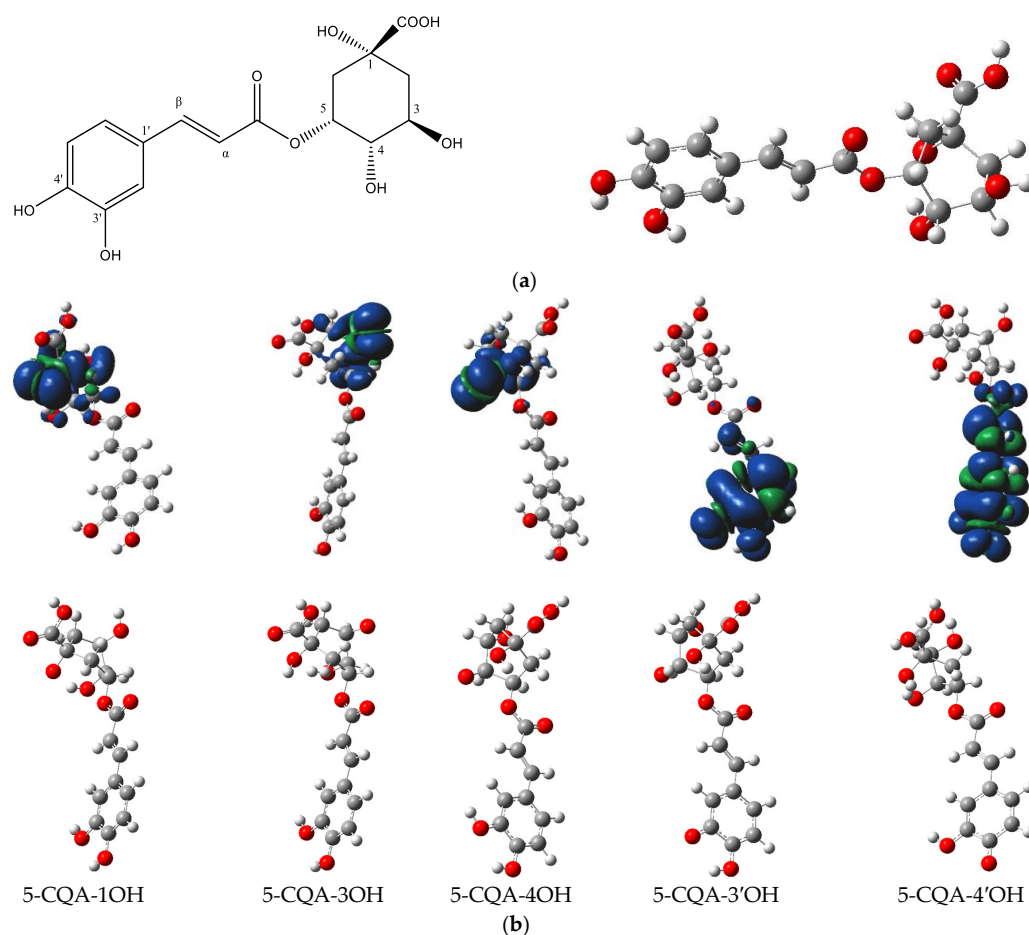


Figure 6. (a) Chemical structure of chlorogenic acid (5-O-caffeoylquinic acid) and its lowest-energy structural conformer; (b) the electron spin density of the optimized structures of 5-O-caffeoylquinic acid radicals.

For the HAT mechanism, 5-CQA-4'OH H-atom abstraction appears to be the favored mechanism for quenching DPPH radical according to the low BDE value (Table 5) and stable electron distribution on the phenolic radical (Figure 6b). For the SET-PT mechanism, the proton transfer step is more highly favored at positions 3' and 4' based on the low PDE values (Table 5). Overall, the SET-PT mechanism at the 5-CQA-3'OH and 5-CQA-4'OH positions appear to be the preferred mechanism for DPPH radical scavenging compared to the HAT and SPLET mechanisms due to the low enthalpy values (Table 5).

4. Discussion

Oxidative stress is pivotal in the pathological outcomes of many diseases, which has been linked to the oxidative damage caused by deleterious ROS on biological molecules [1]. The peel of avocado fruit has been shown to have antioxidant effects [18–20]. In this study, we presented the antioxidant phytochemistry of avocado fruit peel.

The highly reactive nature of biological radicals notably influences their ability to oxidatively damage biomolecules. Plant polyphenols, on the other hand, are known radical scavengers, due to their ability to transfer hydroxyl H-atom and form stable phenoxy radicals [27]. Also, the iron-chelating ability of polyphenols contributes to their antioxidant potential [28]. In the present study, the methanol extract of avocado fruit peel had the highest polyphenol content and consistently had the highest radical scavenging and Fe^{3+} reducing activity relative to the other extracts (Table 1). Although not on the same variety, previous studies have documented the potent radical scavenging effects of the peel's methanol extract [21,29]. Consistent with our study, the methanol extract was more potent than ethyl acetate extract [29]. Among all the fractions from the methanol extract, fraction 8 had the most potent radical scavenging and Fe^{3+} reducing activity (Table 2). The activity correlates with the total phenol content (Table 2), confirming the influence of polyphenols on the radical quenching potential of plants. Supporting documented evidence has also shown correlations between the phenolic contents of avocado peel extract and its radical scavenging and in vitro antioxidant capacity [20,29]. In fact, relative to the pulp and seed, studies have shown that the peel possessed more phenolic content, and thus had stronger radical scavenging and antioxidant capacity [20,30,31].

Previous studies have reported the presence of phenolic acids, including hydroxybenzoic and hydroxycinnamic acids and flavonoids, including anthocyanins, flavanols, flavanones, and flavonols in avocado peel [18–20], which were partly identified in fraction 8 (Table 3). Also, the phenol content of fraction 8 was higher than the flavonoid content (Table 2), which suggests phenolic compounds may have influenced its antioxidant capacity more than the flavonoids. A consistent trend of data has previously been reported in fractions obtained from the methanol and ethyl acetate extracts of peel of an avocado variety grown in Ampelgading, Indonisa [28]. It was shown that the phenolic content contributed more to the radical scavenging and ferric reducing activity of the fractions than the flavonoids content [28]. In the study, 1,2,4-trihidroksiheptadek-12,16-diyne and 1,2,4-trihidroksiheptadek-16-yne-18-ene were identified as the possible antioxidant compounds in the fraction obtained from the methanol and ethyl acetate extracts, respectively [28]. However, this study [28], like other studies [18–20], did not compare the antioxidant activity of the identified compounds with that of the potent extracts or fractions in which they were identified. Thus, the study was not able to ascertain whether the identified compounds influence the antioxidant capacity of the peel extracts or fractions. This suggests a gap in these studies and provides the rationale and relevance of our study.

In our study, there was a predominant presence of chlorogenic and 1-O-caffeoylquinic acid in fraction 8 (Table 3). Chlorogenic acid and derivatives have been reported in avocado peel [Rodríguez-Martínez; Hirasawa]. The data of our study further showed that the radical scavenging activity chlorogenic acid was comparable to that of fraction 8 and ascorbic acid (Table 4 and Figure 2a), suggesting that chlorogenic acid may strongly influence the antioxidant effect of fraction 8. Moreover, chlorogenic acid is a known dietary phenolic acid with antioxidant and anti-inflammatory potentials [32–34]. Further computational DFT

analysis suggests that the favored radical scavenging mechanism of chlorogenic acid is a single electron transfer followed by proton transfer at 3'OH and 4'OH positions (Table 5 and Figure 6).

Furthermore, the radical scavenging potency of fraction 8 and chlorogenic acid (Table 4) may be relevant in their anti-lipid peroxidative attributes. This is because during lipid peroxidation free radicals can oxidatively attack biological lipids, including membrane lipids, compromising cell integrity, and exacerbating oxidative stress [35]. In vitro and in Chang cells, fraction 8 and chlorogenic acid dose-dependently inhibited lipid peroxidation (Table 4 and Figures 2b and 3a). In Chang cells, the anti-lipid peroxidative effect of fraction 8 and chlorogenic acid was accompanied by the modulation of SOD and catalase activities (Figure 3b), which are key antioxidant enzymes. The anti-lipid peroxidative and antioxidant enzyme modulatory effect of chlorogenic acid was comparable to that of ascorbic acid and fraction 8, suggesting chlorogenic acid may notably influence the anti-lipid peroxidative effect of fraction 8. Moreover, the anti-lipid peroxidative and antioxidant enzyme modulatory effect of chlorogenic acid has been documented in animal disease models [36,37].

In LPS-treated RAW 264.7 macrophages, fraction 8 and chlorogenic acid dose-dependently suppressed oxidative stress by suppressing ROS production (Table 4 and Figure 4). This data, together with the potent anti-lipid peroxidative and antioxidant enzyme modulatory action of chlorogenic acid (Figure 3) strongly supports the documented modulatory action of chlorogenic acid on biological redox status [32,33,36,37], and further supports its notable contribution to the antioxidant properties of avocado peel. Furthermore, both fraction 8 and chlorogenic acid dose-dependently suppressed NO production in LPS-treated RAW 264.7 macrophages (Table 4 and Figure 5a) without causing cytotoxicity (Figure 5b). In fact, they outperformed ascorbic acid. Immune myeloid cells, like macrophages, are known to produce more cytokines and NO in response to inflammatory signals [38]. Thus, the inhibitory action of fraction 8 on NO production in RAW 264.7 macrophages induced with inflammation suggests an anti-inflammatory potential. Through the suppressive action on LPS-induced NO production and TNF- α expression in RAW 264.7 macrophages, existing data have shown the anti-inflammatory action of the hydroalcoholic extract of avocado peel [18]. Data of our study further shows that the anti-inflammatory potential of avocado peel may be strongly influenced by the presence of chlorogenic acid, considering the promising suppressive effect of chlorogenic acid on LPS-induced NO production (Table 4 and Figure 5a). Moreover, chlorogenic acid has been reported to potentiate anti-inflammatory action by moderating the production and action of inflammatory mediators, including TNF- α , IL-1 β , IL-6, IL-8, NO, and PGE₂, while modulating signaling processes associated with NF- κ B, MAPK, Nrf2, etc. [34].

5. Conclusions

In this study, a bioassay-guided approach was used to elucidate the antioxidant phytochemistry of avocado peel. Fractionation of the peel's methanol extract yielded a fraction that had promising ability to scavenge free radical, inhibit lipid peroxidation, suppress ROS and NO production and modulate antioxidant enzyme activity. The fraction also showed the predominant presence of chlorogenic acid. Data further showed that chlorogenic acid had antioxidant activities that were comparable to that of the fraction and ascorbic acid. It scavenged DPPH radical through a single electron transfer, followed by proton transfer at its carbons 3'OH and 4'OH positions. Considering that our investigation was bioassay-guided, it is logical to conclude that chlorogenic acid is a bioactive principle that notably influences the antioxidant capacity of avocado fruit peel.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox13040456/s1>.

Author Contributions: G.O.I.: methodology, formal analysis, investigation, data curation, writing—original draft; E.M.N.: methodology, formal analysis, investigation, data curation, writing—review and editing; G.T.T.: methodology, formal analysis, data curation, writing—review and editing; J.N.: methodology, data curation, formal analysis, writing—review and editing; K.P.O.: methodology, data curation, formal analysis, writing—review and editing; S.E.T.: methodology, formal analysis, data curation, writing—review and editing; V.O.F.: methodology, data curation, writing—review and editing; A.M.A.: methodology, data curation, writing—review and editing; O.L.E.: formal analysis, data curation, writing—review and editing; S.S.M.: resources, supervision, writing—review and editing; T.J.M.: resources, writing—review and editing; M.P.S.: resources, writing—review and editing; C.I.C.: conceptualization, resources, supervision, funding acquisition, formal analysis, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: The study was funded by the postgraduate scholarship (reference: MND200429517886) and incentive funding for rated researchers (Grant No: 150945) of the National Research Foundation, South Africa.

Institutional Review Board Statement: The protocol reported in this study was approved by the Faculty Research and Innovation Committee of the Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein on 18 May 2021.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data of this study is available from the corresponding author on request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Jomova, K.; Raptova, R.; Alomar, S.Y.; Alwasel, S.H.; Nepovimova, E.; Kuca, K.; Valko, M. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. *Arch. Toxicol.* **2023**, *97*, 2499–2574. [[CrossRef](#)] [[PubMed](#)]
2. Bardaweel, S.K.; Gul, M.; Alzweiri, M.; Ishaqat, A.; ALSalamat, H.A.; Bashatwah, R.M. Reactive oxygen species: The dual role in physiological and pathological conditions of the human body. *Eurasian J. Med.* **2018**, *50*, 193–201. [[CrossRef](#)] [[PubMed](#)]
3. Hameister, R.; Kaur, C.; Dheen, S.T.; Lohmann, C.H.; Singh, G. Reactive oxygen/nitrogen species (ROS/RNS) and oxidative stress in arthroplasty. *J. Biomed. Mater. Res.* **2020**, *108*, 2073–2087. [[CrossRef](#)] [[PubMed](#)]
4. Mandal, M.; Sarkar, M.; Khan, A.; Biswas, M.; Masi, A.; Rakwal, R.; Agrawal, G.K.; Srivastava, A.; Sarkar, A. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plants— maintenance of structural individuality and functional blend. *Adv. Red. Res.* **2022**, *5*, 100039. [[CrossRef](#)]
5. Jha, N.; Ryu, J.J.; Choi, E.H.; Kaushik, N.K. Generation and role of reactive oxygen and nitrogen species induced by plasma, lasers, chemical agents, and other systems in dentistry. *Oxidative Med. Cell. Longev.* **2017**, *2017*, 7542540. [[CrossRef](#)] [[PubMed](#)]
6. Steinberg, S.F. Oxidative stress and sarcomeric proteins. *Cir. Res.* **2013**, *112*, 393–405. [[CrossRef](#)] [[PubMed](#)]
7. Reczek, C.R.; Chandel, N.S. The two faces of reactive oxygen species in cancer. *Annu. Rev. Cancer Biol.* **2017**, *1*, 79–98. [[CrossRef](#)]
8. Perrone, A.; Giovino, A.; Benny, J.; Martinelli, F. Advanced Glycation End Products (AGEs): Biochemistry, signaling, analytical methods, and epigenetic effects. *Oxidative Med. Cell. Longev.* **2020**, *2020*, 3818196. [[CrossRef](#)] [[PubMed](#)]
9. Birben, E.; Sahiner, U.M.; Sackesen, C.; Erzurum, S.; Kalayci, O. Oxidative stress and antioxidant defense. *World Allergy Organ J.* **2012**, *5*, 9–19. [[CrossRef](#)]
10. Sun, C.; Zhao, C.; Guven, E.C.; Paoli, P.; Simal-Gandara, J.; Ramkumar, K.M.; Wang, S.; Buleu, F.; Pah, A.; Turi, V.; et al. Dietary polyphenols as anti-diabetic agents: Advances and opportunities. *Food Front.* **2020**, *1*, 18–44. [[CrossRef](#)]
11. Afam, I.O.J.; Silungwe, H.; Takalani, T.; Omolola, A.O.; Udeh, H.O.; Anyasi, T.A. Antioxidant-rich natural fruit and vegetable products and human health. *Int. J. Food Prop.* **2021**, *24*, 41–67.
12. Chukwuma, C.I.; Mashele, S.S.; Akuru, E.A. Evaluation of the in vitro α -amylase inhibitory, antiglycation, and antioxidant properties of *Punica granatum* L. (pomegranate) fruit peel acetone extract and its effect on glucose uptake and oxidative stress in hepatocytes. *J. Food Biochem.* **2020**, *44*, e13175. [[CrossRef](#)] [[PubMed](#)]
13. Sha, S.P.; Modak, D.; Sarkar, S.; Roy, S.K.; Sah, S.P.; Ghatani, K.; Bhattacharjee, S. Fruit waste: A current perspective for the sustainable production of pharmacological, nutraceutical, and bioactive resources. *Front. Microbiol.* **2023**, *14*, 1260071. [[CrossRef](#)]
14. Hussain, H.; Mamadalieva, N.Z.; Hussain, A.; Hassan, U.; Rabnawaz, A.; Ahmed, I.; Green, I.R. Fruit Peels: Food Waste as a Valuable Source of Bioactive Natural Products for Drug Discovery. *Curr. Issues Mol. Biol.* **2022**, *44*, 1960–1994. [[CrossRef](#)]
15. Obboh, G.; Odubanjo, V.O.; Bello, F.; Ademosun, A.O.; Oyeleye, S.I.; Nwanna, E.E.; Ademiluyi, A.O. Aqueous extracts of avocado pear (*Persea americana* Mill.) leaves and seeds exhibit anti-cholinesterases and antioxidant activities in vitro. *J. Basic Clin. Physiol. Pharmacol.* **2016**, *27*, 131–140. [[CrossRef](#)]

16. Ferreira, S.M.; Falé, Z.; Santos, L. Sustainability in skin care: Incorporation of avocado peel extracts in topical formulations. *Molecules* **2022**, *27*, 1782. [\[CrossRef\]](#)
17. Araujo, R.G.; Rodríguez-Jasso, R.M.; Ruíz, H.A.; Govea-Salas, M.; Pintado, M.; Aguilar, C.N. Recovery of bioactive components from avocado peels using microwave-assisted extraction. *Food Bioprod. Process.* **2021**, *127*, 152–161. [\[CrossRef\]](#)
18. Tremocoldi, M.A.; Rosalen, P.L.; Franchin, M.; Massarioli, A.P.; Denny, C.; Daiuto, É.R.; Paschoal, J.A.R.; Melo, P.S.; Alencar, S.M. Exploration of avocado by-products as natural sources of bioactive compounds. *PLoS ONE* **2018**, *13*, e0192577. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Rodríguez-Martínez, B.; Ferreira-Santos, P.; Gullón, B.; Teixeira, J.; Botelho, C.; Yáñez, R. Exploiting the potential of bioactive molecules extracted by ultrasounds from avocado peels—Food and nutraceutical applications. *Antioxidants* **2021**, *10*, 1475. [\[CrossRef\]](#)
20. Lyu, X.; Agar, O.T.; Barrow, C.J.; Dunshea, F.R.; Suleria, H.A.R. Phenolic compounds profiling and their antioxidant capacity in the peel, pulp, and seed of Australian grown avocado. *Antioxidants* **2023**, *12*, 185. [\[CrossRef\]](#)
21. Rahman, N.; Sabang, S.M.; Abdullah, R.; Bohari, B. Antioxidant properties of the methanolic extract of avocado fruit peel (*Persea americana* Mill.) from Indonesia. *J. Adv. Pharm. Technol. Res.* **2022**, *13*, 166–170. [\[PubMed\]](#)
22. Motloun, D.M.; Mashele, S.S.; Matowane, G.R.; Swain, S.S.; Bonnet, S.L.; Noreljaleel, A.E.M.; Oyedemi, S.O.; Chukwuma, C.I. Synthesis, characterization, antidiabetic and antioxidative evaluation of a novel Zn (II)-gallic acid complex with multi-facet activity. *J. Pharm. Pharmacol.* **2020**, *72*, 1412–1426. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Mashile, B.; Setlhodi, R.; Izu, G.O.; Erukainure, O.L.; Mashele, S.S.; Makhafola, T.J.; Eze, K.C.; Chukwuma, C.I. Temperature-dependent extraction and chromatographic recovery and characterization of ellagitannins with potent antioxidant and glycemic control properties from “Wonderful” pomegranate peel. *J. Food Sci. Technol.* **2024**, *59*, 408–424. [\[CrossRef\]](#)
24. Marrazzo, P.; Angeloni, C.; Freschi, M.; Lorenzini, A.; Prata, C.; Maraldi, T.; Hrelia, S. Combination of epigallocatechin gallate and sulforaphane counteracts in vitro oxidative stress and delays stemness loss of amniotic fluid stem cells. *Oxidative Med. Cell. Longev.* **2018**, *2018*, 30647811. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Mfotie, E.; Ndemangou, B.; Akinyelu, J.; Munvera, A.M.; Chukwuma, C.I.; Mkounga, P.; Mashele, S.S.; Makhafola, T.J.; McGaw, L.J. In vitro antiproliferative, anti-inflammatory effects and molecular docking studies of natural compounds isolated from *Sarcocephalus pobeguini* (Hua ex Pobeg). *Front. Pharmacol.* **2023**, *14*, 1205414. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Galano, A.; Alvarez-Idaboy, J.R. A computational methodology for accurate predictions of rate constants in solution: Application to the assessment of primary antioxidant activity. *J. Comput. Chem.* **2013**, *34*, 2430–2445. [\[CrossRef\]](#)
27. Chen, J.; Yang, J.; Ma, L.; Li, J.; Shahzad, N.; Kim, C.K. Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Sci. Rep.* **2020**, *10*, 2611. [\[CrossRef\]](#)
28. Scarano, A.; Laddomada, B.; Blando, F.; De Santis, S.; Verna, G.; Chieppa, M.; Santino, A. The chelating ability of plant polyphenols can affect iron homeostasis and gut microbiota. *Antioxidants* **2023**, *12*, 630. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Antasionasti, I.; Riyanto, S.; Rohman, A. Antioxidant activities and phenolics contents of avocado (*Persea americana* Mill.) peel in vitro. *J. Med. Plant Res.* **2017**, *11*, 55–61.
30. Rojas-García, A.; Villegas-Aguilar, M.d.C.; García-Villegas, A.; Cádiz-Gurrea, M.d.I.L.; Fernández-Ochoa, Á.; Fernández-Moreno, P.; Arráez-Román, D.; Segura-Carretero, A. Characterization and biological analysis of avocado seed and peel extracts for the development of new therapeutical strategies. *Biol. Life Sci. Forum* **2022**, *18*, 9. [\[CrossRef\]](#)
31. Hirasawa, M.; Shimura, K.; Shimizu, A.; Mura, K.; Tokue, C.; Arai, S. Quantification and functional analysis of dietary fiber and polyphenols in avocado. *J. Jpn. Soc. Food Sci. Technol.* **2008**, *55*, 95–101. [\[CrossRef\]](#)
32. Liang, N.; Kitts, D.D. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients* **2015**, *8*, 16. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Wang, L.; Pan, X.; Jiang, L.; Chu, Y.; Gao, S.; Jiang, X.; Zhang, Y.; Chen, Y.; Luo, S.; Peng, C. The biological activity mechanism of chlorogenic acid and its applications in food industry: A review. *Front. Nutr.* **2022**, *9*, 943911. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Huang, J.; Xie, M.; He, L.; Song, X.; Cao, T. Chlorogenic acid: A review on its mechanisms of anti-inflammation, disease treatment, and related delivery systems. *Front. Pharmacol.* **2023**, *14*, 1218015. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Petrovic, S.; Arsic, A.; Ristic-Medic, D.; Cvetkovic, Z.; Vucic, V. Lipid peroxidation and antioxidant supplementation in neurodegenerative diseases: A review of human studies. *Antioxidants* **2020**, *9*, 1128. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Zhou, J.; Ashoori, F.; Suzuki, S.; Nishigaki, I.; Yagi, K. Protective effect of chlorogenic acid on lipid peroxidation induced in the liver of rats by carbon tetrachloride or ⁶⁰Co-irradiation. *J. Clin. Biochem. Nutr.* **1993**, *15*, 119–125. [\[CrossRef\]](#)
37. Wang, D.; Tian, L.; Lv, H.; Pang, Z.; Li, D.; Yao, Z.; Wang, S. Chlorogenic acid prevents acute myocardial infarction in rats by reducing inflammatory damage and oxidative stress. *Biomed. Pharm.* **2020**, *132*, 110773. [\[CrossRef\]](#)
38. Palmieri, E.M.; McGinity, C.; Wink, D.A.; McVicar, D.W. Nitric oxide in macrophage immunometabolism: Hiding in plain sight. *Metabolites* **2020**, *10*, 429. [\[CrossRef\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.



Article

Epicatechin Isolated from *Litchi chinensis* Sonn. (Litchi) Fruit Peel Ethyl Acetate Extract Modulated Glucose Uptake in Chang Cells and Suppressed ROS Production in RAW 264.7 Macrophages

Gloria O. Izu ^{1,2}, Nomonde P. Mapasa ^{1,2}, Jennifer Namboozee ³, Maria S. Chukwuma ³, Emmanuel Mfotie Njoya ², Gaetan T. Tabakam ², Susanna L. Bonnet ³, Tshepiso J. Makhafole ², Samson S. Mashele ² and Chika I. Chukwuma ^{2,*}

¹ Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein 9301, Free State, South Africa; 221039621@stud.cut.ac.za (G.O.I.); 224107123@cut.ac.za (N.P.M.)

² Centre for Quality of Health and Living (CQHL), Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein 9301, Free State, South Africa; enjaya@cut.ac.za (E.M.N.); tgaetan@cut.ac.za (G.T.T.); jmakhafole@cut.ac.za (T.J.M.); smashele@cut.ac.za (S.S.M.)

³ Department of Chemistry, Faculty of Natural and Agricultural Sciences, University of the Free State, Bloemfontein 9301, Free State, South Africa; 2020280521@ufs4life.ac.za (J.N.); 2017373891@ufs4life.ac.za (M.S.C.); bonnetsl@ufs.ac.za (S.L.B.)

* Correspondence: cchukwuma@cut.ac.za



Citation: Izu, G.O.; Mapasa, N.P.; Namboozee, J.; Chukwuma, M.S.; Njoya, E.M.; Tabakam, G.T.; Bonnet, S.L.; Makhafole, T.J.; Mashele, S.S.; Chukwuma, C.I. Epicatechin Isolated from *Litchi chinensis* Sonn. (Litchi) Fruit Peel Ethyl Acetate Extract Modulated Glucose Uptake in Chang Cells and Suppressed ROS Production in RAW 264.7 Macrophages. *Antioxidants* **2024**, *13*, 1233. <https://doi.org/10.3390/antiox13101233>

Academic Editor: Julia González-Álvarez

Received: 12 September 2024

Revised: 8 October 2024

Accepted: 10 October 2024

Published: 14 October 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Bioactive flavonoid epicatechin has been reported in the peel of litchi fruit but isolated from its hydroalcoholic extracts. This study isolated epicatechin with cellular glucose uptake modulatory and ROS production inhibitory properties from the ethyl acetate (EtOAc) extract using a bioassay-guided approach. The fruit peel was defatted with hexane and sequentially extracted using dichloromethane (DCM), EtOAc, methanol (MeOH) and water. In vitro phytochemical models, namely antioxidant (Fe^{3+} reducing, radical scavenging and anti-linoleic acid peroxidative) and glycaemic control (α -glucosidase and α -amylase inhibitory and glucose uptake modulatory), were employed for the bioassay-guided isolation, while the isolated compound was characterised using NMR and mass spectrometry and assessed for dose-dependent inhibition of α -glucosidase and lipopolysaccharide (LPS)-induced cellular ROS production, as well as modulation of cellular glucose uptake. Relative to the other extracts, the EtOAc extract had appreciable phenol and flavonoid contents, which perhaps influenced its potent anti-lipid peroxidative (65.0%) and α -glucosidase inhibitory (52.4%) effects. The α -glucosidase inhibitory potency of the fractions (1–8) from the EtOAc extracts correlated with their flavonoid contents, with fraction 5 outperforming other fractions. The fraction comprised a pool of fractions obtained from the DCM:MeOH:water (7:3:0.281 v/v/v) solvent system. LC-MS revealed the predominant presence of epicatechin in fraction 5, which was later isolated from one of the sub-fractions (sub-fraction 4) of fraction 5. This sub-fraction had stronger anti-lipid peroxidative (65.5%), α -glucosidase inhibitory (65.8%) and glucose uptake modulatory (38.2%) effects than the other sub-fractions from fraction 5, which could have been influenced by the isolated epicatechin. Moreover, the isolated epicatechin inhibited α -glucosidase ($\text{IC}_{50} = 35.3 \mu\text{M}$), modulated cellular glucose uptake ($\text{EC}_{50} = 78.5 \mu\text{M}$) and inhibited LPS-induced ROS production in RAW 264.7 macrophages in a dose-dependent fashion [$\text{IC}_{50} = 18.9 \mu\text{M}$; statistically comparable ($p > 0.05$) to ascorbic acid, $\text{IC}_{50} = 9.57 \mu\text{M}$]. Epicatechin from litchi peel EtOAc extract could potentiate glucose uptake modulatory, α -glucosidase inhibitory and ROS suppressive capacities, which could be influential in the use of litchi fruit peel for managing diabetes and associated oxidative damage.

Keywords: antioxidant; epicatechin; glucose uptake; isolation; litchi peel

1. Introduction

Diabetes remains a global health concern as it detrimentally affects the socio-economic sphere of many countries around the world. This has been aggravated by its ever-increasing prevalence, projected to increase from 537 million adults in 2021 to 783 million adults in 2045 [1]. The disease is classically characterised by high blood glucose caused by abnormal nutrient metabolism. Type 2 diabetes (T2D) remains the most prevalent type of diabetes [1], possibly because it is influenced by lifestyle-related factors, including dieting, weight gain and physical inactivity [2]. Insulin resistance is the main pathologic condition in T2D. Typically, it causes impaired metabolism of glucose. This includes drastically reduced uptake of circulating glucose into active cells of peripheral tissues due to the non-responsiveness of these cells to insulin signalling, which contributes to hyperglycaemia [3].

Hyperglycaemia-induced oxidative stress is pivotal in developing diabetic complications due to oxidative damages caused by reactive oxygen species (ROS) produced during persistent hyperglycaemia [4]. Notably, there is an elevated generation of superoxide radicals in respiring cells during hyperglycaemia [4]. Elevated radical production can trigger lipid peroxidation. ROS can oxidatively damage the lipid bilayer of cells through lipid peroxidation, comprising the integrity of the cells [4]. Moreover, lipid peroxidation processes can lead to the generation of more deleterious radical by-products, which can worsen oxidative stress. Furthermore, persistent hyperglycaemia can increase the rate of glycation of other biomolecules. This leads to the generation of advanced glycation end products (AGEs), biomarkers implicated in degenerative diseases like diabetes [4].

While there are many medications for managing diabetes and associated oxidative complications, dietary phytochemicals are increasingly becoming popular supplements to mitigate oxidative stress and manage metabolic diseases [5]. Many fruits are believed to contain antioxidant phenolics that influence their health benefits [6]. *Litchi chinensis* Sonn. (Sapindaceae) is a tropical and subtropical fruit with high commercial value due to its global consumption. It is known to have nutritional value and medicinal benefits [7]. Studies have shown that the fruit has antioxidant, anti-hyperglycaemic, anti-hypertensive, anti-obesogenic, anti-dyslipidaemic and hepatoprotective potentials [7]. Due to its commercial value, enormous amounts of by-products, including the peel and seeds, are generated from its processing, which contains bioactive phenolic compounds in their free and bound forms [8]. Thus, the fruit's wastes could be of medicinal relevance.

In the context of antidiabetic potential, a previous review [9] has shown that more studies have been conducted on the seed of litchi fruit than the peel, warranting the need for more studies to probe the antidiabetic potential of the peel. The few antidiabetic studies that have been conducted suggest that fruit peel has glycaemic control potential. A study has shown that hydroalcoholic extracts of litchi peel have mild and strong inhibitory activity on α -amylase and α -glucosidase, respectively [10], suggesting its potential to suppress postprandial glycaemia. In another study, the peel flour and procyanidin-rich fraction from the peel reduced blood glucose and improved blood lipid profile in high-fat-diet-fed rats and mice [11,12]. A recent study further showed that litchi peel ethanol extract ameliorated T2D pathologies in db/db mice by reducing blood glucose, hepatic gluconeogenesis, oxidative stress and associated inflammatory responses [13]. Phytochemical assessments suggest that several key flavonoids, including catechins, procyanidins, proanthocyanidins, quercetin, rutin and their derivatives, may be influential in the antioxidant and other pharmacological properties of litchi peel [14–16].

The trend of previous diabetes-related pharmacological studies on litchi peel suggests that the focus has mostly been on the hydroalcoholic extracts, with limited studies on other extracts, such as the ethyl acetate extract [9]. Solvent extraction using ethyl acetate allows hydrogen bond formation with carboxyl and hydroxyl groups. It thus can result in the extraction of medium-polar to polar phenolics and/or flavonoids [17], which could influence potent medicinal properties. In fact, documented qualitative evidence has shown that litchi peel hydroalcoholic and ethyl acetate extracts have relatively similar proportions of phenolic compounds and flavonoids [18]. Concomitant pharmacological evidence in diabetic

rats further suggests that the blood glucose lowering and antioxidant effect of litchi peel ethyl acetate extract outperforms that of the hydroalcoholic extract [18], suggesting that the ethyl acetate extract of litchi peel could also be a source of flavonoids with antioxidant and glycaemic control potential. However, this has not been explored and thus remains elusive. Previous studies reporting the isolation of some bioactive flavonoids (proanthocyanidin B4, proanthocyanidin B2 and epicatechin) from litchi peel were from the hydroalcoholic extract and not the ethyl acetate extract [14,15]. Thus, there is a need to explore litchi peel ethyl acetate extract as a source of key bioactive flavonoids. Therefore, in this study, we, for the first time, isolated a key bioactive flavonoid (epicatechin) from litchi peel ethyl acetate extract using an antioxidant and glycaemic control assay-guided approach, thus providing an alternative optimum approach to recovering bioactive epicatechin from litchi peel and more insight into the antidiabetic and antioxidant phytochemistry of litchi peel.

2. Materials Methods

2.1. Litchi Fruit Peel and Solvent Extraction

Litchi fruit (McLean's Red cultivar) was purchased from a local fruit and vegetable store in Bloemfontein, South Africa. The fruit was washed with clean water. The peel was removed and oven-dried (Model No. 072160, Prolab instrument, Sep Sci., Johannesburg, South Africa) at 30 °C. The dried peel was pulverised. Approximately 300 g of the pulverised dried peel was defatted with 1.5 L of hexane and then sequentially extracted using 1.5 L of dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH) and water in order of increasing polarity. The extraction was done for 48 h at room temperature on an orbital shaker [OrbiShake, Model 262, Labotec 106 (Pty) Ltd., Johannesburg, South Africa] set at 125 rpm. The extraction was performed twice for each solvent. The DCM, EtOAc and MeOH extracts were concentrated using a Buchi 108 Rotavapor® R-300 [Labotec (Pty) Ltd., Johannesburg, South Africa] and dried under a fume hood. The water extract was recovered by freeze drying (Martin Christ Alpha 1–2 LDplus Freeze Dryer 110, Separations, Johannesburg, South Africa). The extracts were weighed, transferred into clean screw-cap vials and stored at −20 °C.

2.2. Screening the Phenolic, Flavonoid, Antioxidant and Glycaemic Control Properties of Extracts

The extracts were screened at a concentration of 55 µg/mL. The total phenol and flavonoid contents were measured using previously described methods [19]. The total phenol and flavonoid contents were expressed in equivalents of gallic acid (mg/g GAE) and quercetin (mg/g QE), respectively.

For the antioxidant screening, the Fe³⁺ reducing (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and NO radicals scavenging and anti-linoleic acid peroxidative models were used, which were performed according to the protocols reported previously [19–21]. Ascorbic acid and Trolox were used as the positive control drugs.

For the glycaemic control screening, the extracts were tested for their ability to inhibit the activity of α-glucosidase and α-amylase, which was performed by adopting previously reported protocols [20]. Acarbose was used as the positive control drug.

2.3. Bioassay-Guided Fractionation of the Ethyl Acetate Extract and Isolation/Purification of Pure Compounds

A flow diagram of the fractionation and isolation protocol is presented in Figure 1. The extract was subjected to a silica gel 60 (0.040–0.063 mm Merck) gradient column chromatography, starting with a solvent system (DCM:MeOH:water; 9:1:0.125 v/v/v), which was pre-determined by thin layer chromatography (TLC). The column was run with a decreasing volume of DCM, an increasing volume of MeOH and water, as shown in the Supplementary Materials (Table S1). The fractions obtained were pooled into 8 main fractions (Table S1) based on their TCL profiles. The fractions were identified as fractions 1 to 8.

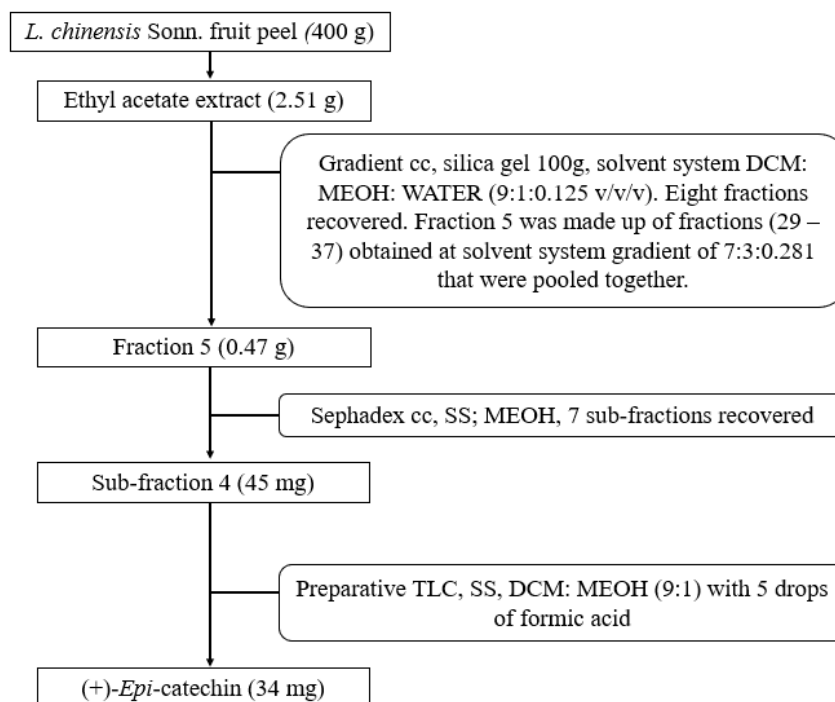


Figure 1. Schematic representation of the isolation of compounds from the ethyl acetate extract of litchi peel.

The total phenol and flavonoid contents, as well as the antioxidant (FRAP, DPPH and ABTS radicals scavenging activity) and glycaemic control (α -glucosidase and α -amylase inhibition) potentials of the fractions were measured at a concentration of 35 μ g/mL according to protocols previously reported [19,20].

Based on the bioactivity results obtained, fraction 5, which comprises a pool of fractions (29–37) obtained from a solvent system gradient of 7:3:0.281 v/v/v (DCM:MeOH:water), was selected for further fractionation using a Sephadex (Merck, Johannesburg, South Africa) column with 100% methanol as the solvent system. Also, LC-MS was used to chemically profile fraction 5 according to a protocol reported previously [22]. Using the accurate masses and extracted ions, the compounds in the fraction were tentatively determined and semi-quantified (mg/L) relative to catechin calibration and according to the extracted ions.

Seven sub-fractions were obtained from fraction 5 after separation in a Sephadex column. The in vitro radical (DPPH and ABTS) scavenging, anti-linoleic acid peroxidative, α -glucosidase inhibitory and cellular glucose uptake modulatory activities of the sub-fractions were measured at 20 μ g/mL using methods reported in previous studies [20,21].

Chang cells were used to measure the cellular glucose uptake modulatory activity of the sub-fractions, and a previous protocol [20] was adopted after some modifications. Chang cells (ATCC[®] CCL-13[™]; American Type Culture Collection Rockville, MD, USA) were cultured in a CO₂ incubator using DMEM-high glucose media supplemented with 10% FBS (Separations, Johannesburg, South Africa). At confluence, the cells were seeded into a 48-well plate at a density of 15,000 cells per well. After 24 h of incubation, the spent media was replaced with 350 μ L of fresh media containing the sub-fractions (20 μ g/mL in incubation medium). After 24 h of incubation, the media was aspirated, and the cells were washed with PBS. Next, 350 μ L of RPMI media (diluted to 8 mM glucose concentration) containing the sub-fractions (20 μ g/mL in incubation medium) was added to the wells. Designated wells were used as the media (containing media without cells) and control (containing media with untreated cells) groups. The plate was incubated for 3 h. Thereafter, 20 μ L aliquot from each well was diluted 24 times with distilled water and glucose concentration (in mg/mL) was measured in the diluted aliquots against a glucose standard curve ($y = 9.766x + 0.0585$) using a Glucose (GO) Assay Kit (Merck, Johannesburg, South

Africa), which was then converted to concentration in millimolar (mM). The glucose uptake activity (%) of the control and test groups was computed relative to the media group using the following formula:

$$\text{Glucose uptake activity(\%)} = \frac{GC_{\text{media}} - GC_{\text{control or test}}}{GC_{\text{media}}} \times 100$$

where “GC” is the glucose concentration in millimolars (mMs).

The glucose uptake modulatory activity of the sub-fractions was computed relative to the control by subtracting the glucose uptake activity of the control from that of each fraction.

Based on the bioactivity data obtained from the subfractions, subfraction 4 was further subjected to preparative TLC (20 × 20 cm glass-backed, 0.25 mm silica plates) for the isolation of bioactive compound(s). The solvent system used was DCM: MEOH (9:1) with 5 drops of formic acid. The UV-active substances were scraped off, washed with acetone and methanol consecutively and recovered by filtration.

2.4. Nuclear Magnetic Resonance (NMR) Spectroscopy and Mass Spectrometry (MS)

The ^1H and ^{13}C NMR spectra were acquired using a Bruker Avance 400 MHz spectrometer. Deuterated DMSO solvent was used, and the working temperature was 25 °C. For ^1H resonances (0 ppm), tetramethylsilane (TMS) was employed as an internal standard. The standard pulse sequences and processing macros given in the original program were used for advanced and two-dimensional spectra such as ^{13}C APT/DEPT and 2D COSY, NOESY, HSQC and HMBC.

The compound(s) were subjected to low-resolution mass spectrometry. The MS experiment used an MD Sciex 3200 QTrap equipped with an electrospray (Turbo-ion spray) ionisation source.

2.5. Antioxidant and Glycaemic Control Examination of Isolated Compound

The isolated compound was assessed for dose-dependent antioxidant and glycaemic control potential using the following experimental models.

2.5.1. Measuring the Inhibition of Lipopolysaccharide (LPS)-Induced ROS Production in RAW 264.7 Cells

The protocol reported in a recent study [22] was adopted for this assay after adding some modifications. RAW 264.7 macrophages [American Type Culture Collection (ATCC) Rockville, MD, USA; ATCC No.: TIB-71™] were cultured in a CO₂ incubator (EC 160, Nüve; Separations, Johannesburg, South Africa) using RPMI 1640 medium supplemented with 10% FBS under standard culture conditions. At appreciable confluence, 10,000 cells were seeded into the wells of a black/clear-bottom 96-well plate. The plate was incubated for 24 h before treating the cells with increasing concentrations (8.75, 17.5, 35, 70 and 140 µM) of the isolated compound and ascorbic acid (positive control). The treatment lasted for 2 h. Designated wells were used as the control and negative control groups, which were cells treated with water or DMSO (≤0.5%). After the 2 h treatment period elapsed, LPS (200 ng/mL in final incubation volume) was introduced into all the wells except those labelled as the control group. Rather, an equivalent volume of the LPS solvent was added to the control group wells. The plate was incubated for an additional 24 h. Thereafter, the cells were washed with PBS and exposed to 10 µM (dissolved in FBS-free culture medium) 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Merck, Johannesburg, South Africa) fluorescent probe for 30 min, which was performed in the dark. After the incubation period elapsed, the DCFH-DA-containing medium in the wells was replaced with PBS, and the fluorescence intensity of each well was measured at 485 nm (excitation) and 535 nm (emission) using a SpectraMax iD3 multi-mode microplate reader (Molecular Devices, San Jose, USA). Also, the images of the cells were visualised using a Leica DM IL LED inverted

fluorescent microscope (Separations, Johannesburg, South Africa). The capacity of the compound or ascorbic acid to inhibit ROS production was computed as follows:

$$\text{Inhibition (\%)} = \frac{(F_{\text{Negative control}} - F_{\text{Normal control}}) - (F_{\text{Test}} - F_{\text{Normal control}})}{(F_{\text{Negative control}} - F_{\text{Normal control}})} \times \frac{100}{1}$$

where “F” is the fluorescence intensity.

2.5.2. Measuring the α -Glucosidase Inhibitory Potential of Isolated Compound

The dose-dependent α -glucosidase inhibitory potential of the isolated compound was measured according to a previously reported method [20] and was compared to that of acarbose. The isolated compound and acarbose were tested at the following concentrations: 3.75, 7.5, 15, 30 and 60 μM .

2.5.3. Measuring the Glucose Uptake Activity of Isolated Compound in Chang Cells

The glucose uptake activity of the isolated compound was measured according to the glucose uptake protocol reported in Section 2.3 above. The glucose uptake activity of the isolated compound was tested at increasing concentrations (10, 20, 40 and 80 μM) and was compared to the activity of a 2 μM Metformin solution (positive control).

2.6. Data and Statistical Analysis

The data obtained were in triplicate analysis and were reported as the mean $\pm$ standard deviation. The IBM SPSS Statistics (Windows version 29.0) software was used to compute statistical significance ($p < 0.05$) when comparing the data ($n = 3$) between groups. The one-way ANOVA and Tukey post hoc were adopted for multiple comparative analyses.

The IC_{50} and EC_{50} values were computed from a non-linear fit of the transformed (Log10) tested concentrations versus the inhibitory or modulatory activities using GraphPad Prism 7 (Windows Version) software.

3. Results and Discussion

The antidiabetic and antioxidant potential of litchi peel has been documented [9–13]. However, these studies and the isolation of bioactive flavonoids (epicatechin and proanthocyanidins) from litchi peel have mostly been done on the hydroalcoholic extracts [9–15]. Documented evidence suggests that the ethyl acetate extract of litchi peel may possess stronger in vivo antidiabetic and antioxidant potential than the hydroalcoholic extract [18] and may be a source of the key bioactive flavonoids in litchi peel. However, the ethyl acetate extract of litchi peel has not been explored in this context. In this study we used bioassay-guided isolation to demonstrate for the first time that the ethyl acetate extract of litchi peel can be an alternative source to recover epicatechin with cellular glucose uptake modulatory and ROS production suppressive capacities.

3.1. Phytochemical and Bioactive Profiles of the Peel Extracts

Extraction of the peel with DCM, EtOAc, MeOH and water yielded 17.3 (5.74%), 2.51 (0.84%), 63.1 (21.0%) and 12.6 g (4.20%) of extracts, respectively. The EtOAc extract had the highest ($p < 0.05$) total phenol content. In contrast, the MeOH and EtOAc extracts had significantly ($p < 0.05$) higher total flavonoid contents than the DCM and water extracts (Table 1). The rich phenolic and flavonoid profile of the EtOAc extract could be linked to its ability to form hydrogen bonds with carboxyl and hydroxyl groups. It thus can extract medium polar to polar phenolics and/or flavonoids [17]. Perhaps the phenolic and flavonoid profile of the EtOAc extract also influenced its FRAP, radical scavenging and anti-linoleic acid peroxidative activities, which were stronger than those of the other extracts and statistically comparable ($p > 0.05$) to those of ascorbic acid and/or Trolox (Table 1). Phenolics have the ability to form stable phenoxy radicals, which influences their ability to scavenge radicals and chelate metals, making them good antioxidants [23,24]. Moreover, qualitative

phytochemical analysis of litchi peel EtOAc extract and hydroalcoholic extracts has shown the presence of phenolic compounds and flavonoids in a relatively similar fashion and could be influential in the potent radical scavenging, FRAP and anti-lipid peroxidative activities demonstrated by the ethyl acetate and methanol extracts (Table 1) [14–16].

Table 1. Total phenol and flavonoid contents and in vitro Fe^{3+} reducing, radical scavenging anti-linoleic acid peroxidative and enzyme inhibitory activities of crude extracts at 55 $\mu\text{g}/\text{mL}$.

Extracts and Standards	TPC (mg/g GAE)	TFC (mg/g QE)	FRAP (mg/g GAE)	DPPH (%)	ABTS (%)	NO (%)	AnLP (%)	AGI (%)	AAI (%)
DCM	39.7 $\pm$ 1.35 ^a	35.3 $\pm$ 9.71 ^a	151 $\pm$ 5.77 ^a	46.1 $\pm$ 5.07 ^a	43.6 $\pm$ 0.43 ^b	5.63 $\pm$ 2.77 ^a	33.6 $\pm$ 6.28 ^a	5.10 $\pm$ 3.54 ^a	9.84 $\pm$ 4.64 ^a
EtOAc	435 $\pm$ 22.4 ^c	109.5 $\pm$ 12.1 ^c	600 $\pm$ 54.7 ^c	83.3 $\pm$ 2.01 ^b	96.6 $\pm$ 0.26 ^d	15.0 $\pm$ 5.22 ^b	65.0 $\pm$ 3.48 ^b	52.4 $\pm$ 4.63 ^d	33.7 $\pm$ 7.42 ^{bc}
MeOH	152 $\pm$ 2.41 ^b	126 $\pm$ 13.0 ^c	381 $\pm$ 10.5 ^b	44.8 $\pm$ 8.55 ^a	81.9 $\pm$ 0.89 ^c	10.0 $\pm$ 0.68 ^{ab}	35.9 $\pm$ 3.68 ^a	34.1 $\pm$ 3.14 ^c	13.8 $\pm$ 5.16 ^a
Water	55.9 $\pm$ 1.75 ^a	68.9 $\pm$ 3.32 ^b	179 $\pm$ 11.9 ^a	36.1 $\pm$ 1.56 ^a	23.1 $\pm$ 1.10 ^a	22.2 $\pm$ 1.21 ^c	28.0 $\pm$ 4.65 ^a	17.6 $\pm$ 5.18 ^b	25.7 $\pm$ 4.09 ^{ab}
Asc	N/A	N/A	879 $\pm$ 12.0 ^d	45.3 $\pm$ 5.03 ^a	97.9 $\pm$ 0.06 ^{de}	4.41 $\pm$ 1.82 ^a	ND	N/A	N/A
Trolox	N/A	N/A	657 $\pm$ 16.9 ^c	91.9 $\pm$ 3.49 ^b	99.3 $\pm$ 1.10 ^e	13.5 $\pm$ 2.05 ^b	84.1 $\pm$ 5.12 ^c	N/A	N/A
Aca	N/A	N/A	N/A	N/A	N/A	N/A	N/A	88.3 $\pm$ 1.31 ^e	43.6 $\pm$ 11.1 ^c

“AAI”, α -amylase inhibitory activity; “ABTS”, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; “Aca”, acarbose; “AGI”, α -glucosidase inhibitory activity; “AnLP”, anti-linoleic acid peroxidation activity; “Asc”, ascorbic acid; “DCM”, dichloromethane extract; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; “FRAP”, Fe^{3+} reducing antioxidant power; “EtOAc”, ethyl acetate extract; “GAE”, gallic acid equivalent; “MeOH”, methanol extract; “N/A”, not applicable; “ND”, not determined; “NO”, nitric oxide radical scavenging activity; “QE”, quercetin equivalent; “TFC”, total flavonoid content; “TPC”, total phenol content. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Compared to the other extracts, the EtOAc extract has the highest ($p < 0.05$) α -glucosidase and α -amylase inhibitory activities (Table 1). The MeOH has moderate α -glucosidase inhibitory activity. The EtOAc and MeOH extracts show a stronger inhibitory activity on α -glucosidase than α -amylase, which is consistent with a recent report, where litchi peel hydroalcoholic extracts showed more potent inhibition on α -glucosidase than α -amylase [10]. The enzyme inhibitory effect of the polar extracts of litchi peel has been linked to the presence of notable flavonoids such as quercetin glycosides, proanthocyanidin, catechins and derivatives, etc. [10], which are also known to exert enzyme inhibitory action [25]. α -Glucosidase inhibitors exert glycaemic control by reducing postprandial blood glucose [26], reiterating the anti-hyperglycaemic potential of litchi peel.

3.2. Phytochemical and Bioactive Profiles of the Fractions and Sub-Fractions from the EtOAc Peel Extract

Gradient column chromatography yielded fractions that were pooled into eight major fractions (fractions 1–8) based on their TLC profiles (Table S2). Although fraction 5 has the lowest total phenol content, its flavonoid content is significantly ($p < 0.05$) higher than other fractions (Table 2), which suggests that this fraction may contain some of the key bioactive flavonoids reported in litchi peel [10,14–16].

In fact, LC-MS analysis of fraction 5 showed the notable presence of epicatechin, among other flavonoids (Table 3 and Figure 2), which may have influenced its more robust ($p < 0.05$) α -glucosidase and α -amylase inhibitory activity relative to the other fractions (Table 2). Moreover, the enzyme inhibitory activity of epicatechin and derivatives has been documented [27–29]. Fraction 5 also showed appreciable ABTS radical scavenging activity (Table 2) and thus was selected for further fractionation and isolation of bioactive flavonoids.

Table 2. Total phenol and flavonoid contents and in vitro Fe³⁺ reducing, radical scavenging and enzyme inhibitory activities of fractions from the crude ethyl acetate extract at 35 µg/mL.

Fractions and Standards	TPC (mg/g GAE)	TFC (mg/g QE)	FRAP (mg/g GAE)	DPPH (%)	ABTS (%)	AGI (%)	AAI (%)
F1	361 ± 66.0 ^{de}	42.2 ± 8.43 ^b	167 ± 3.08 ^d	40.7 ± 6.98 ^{bc}	80.6 ± 1.42 ^a	22.0 ± 4.44 ^a	14.1 ± 4.98 ^{ab}
F2	322 ± 55.2 ^d	71.7 ± 5.65 ^c	166 ± 3.12 ^d	34.7 ± 4.67 ^{bc}	80.1 ± 1.73 ^a	37.7 ± 5.31 ^{bc}	15.1 ± 3.19 ^{ab}
F3	220 ± 19.5 ^{bc}	73.5 ± 8.55 ^c	124 ± 16.5 ^b	14.9 ± 1.30 ^a	77.9 ± 6.00 ^a	49.3 ± 8.13 ^{cd}	22.9 ± 9.33 ^b
F4	195 ± 19.7 ^{ab}	70.8 ± 16.0 ^c	78.3 ± 12.2 ^a	27.8 ± 6.27 ^{ab}	97.6 ± 2.22 ^d	37.5 ± 5.98 ^{bc}	7.20 ± 3.57 ^a
F5	133 ± 25.8 ^a	104 ± 6.11 ^d	122 ± 6.70 ^b	28.3 ± 5.14 ^b	98.2 ± 1.17 ^d	68.1 ± 1.51 ^e	36.7 ± 4.86 ^c
F6	287 ± 4.33 ^{cd}	54.1 ± 7.50 ^{bc}	166 ± 1.36 ^d	39.4 ± 8.08 ^{bc}	98.0 ± 0.06 ^d	31.7 ± 7.40 ^{ab}	6.39 ± 1.82 ^a
F7	422 ± 3.88 ^e	18.9 ± 2.93 ^a	179 ± 2.05 ^{de}	43.1 ± 1.05 ^c	97.4 ± 2.21 ^d	18.4 ± 1.87 ^a	14.6 ± 2.30 ^{ab}
F8	186 ± 16.4 ^{ab}	18.2 ± 2.12 ^a	145 ± 4.87 ^c	38.2 ± 0.73 ^{bc}	96.9 ± 1.08 ^{cd}	21.9 ± 5.52 ^a	10.8 ± 3.74 ^{ab}
Asc	N/A	N/A	290 ± 4.36 ^f	41.4 ± 1.89 ^c	90.3 ± 1.88 ^{bc}	N/A	N/A
Trolox	N/A	N/A	192 ± 3.93 ^e	79.0 ± 4.13 ^d	87.0 ± 1.50 ^{ab}	N/A	N/A
Aca	N/A	N/A	N/A	N/A	N/A	65.8 ± 2.76 ^e	39.9 ± 3.45 ^c

“AAI”, α-amylase inhibitory activity; “ABTS”, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; “Aca”, acarbose; “AGI”, α-glucosidase inhibitory activity; “Asc”, ascorbic acid; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; “FRAP”, Fe³⁺ reducing antioxidant power; “GAE”, gallic acid equivalent; “N/A”, not applicable; “QE”, quercetin equivalent; “TFC”, total flavonoid content; “TPC”, total phenol content. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Table 3. LC-MS data of fraction 5.

Identified Compounds	Ontology	ART (min)	AMz	Total Score	Peak Height	Conc. (mg/L)
Procyanidin B5	Biflavonoids and polyflavonoids	8.241	577.13507	7.6791	1280	0.3
Pseudolaroside B;(-)-Pseudolaroside B	Hydrolysable tannins	8.802	329.0878	7.4069	2855	0.7
Butyl 3-O-caffeoylquinic acid;Syringic acid;Syringin	Quinic acids and derivatives	9.147	409.15076	7.3007	3121	0.8
4-O-beta-glucoside	Hydrolysable tannins	9.722	359.09879	7.349	5648	1.4
Epicatechin	Catechins	12.133	289.07214	8.0017	370,407	94.5
Kievitone hydrate	8-prenylated isoflavanones	13.707	373.13028	4.7465	14,903	3.8
Garcimangosone D	Phenolic glycosides	14.2	391.1037	7.3375	4686	1.2
5-hydroxy-2-(4-hydroxyphenyl)-7-[[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]-3,4-dihydro-2H-1-benzopyran-4-one	Flavonoid-7-O-glycosides	15.163	433.11365	7.5754	1137	0.3
Garcimangosone D	Phenolic glycosides	15.254	391.1037	7.0551	1280	0.3
Polydine	Flavonoid-7-O-glycosides	16.175	421.11395	7.3603	4486	1.1
Isolariciresinol 9'-O-beta-D-glucoside	Lignan glycosides	16.435	521.20264	7.1728	7835	2.0
Quercetin 3-galactoside	Flavonoid-3-O-glycosides	16.659	463.0878	7.7967	16,029	4.1
Aspalathin	2'-Hydroxy-dihydrochalcones	16.834	451.12402	7.1687	7039	1.8
Aurasperone C	Naphthopyranones	16.843	591.15076	7.5058	27,229	6.9
Isolariciresinol 9'-O-beta-D-glucoside	Lignan glycosides	16.97	521.20251	6.0419	25,518	6.5
Aurovertin G	C-glycosyl compounds	17.308	491.19247	6.3281	50,720	12.9
Hovenitin I	Epigallocatechins	17.492	333.06146	8.0014	9169	2.3
Avicularin	Flavonoid-3-O-glycosides	17.611	433.07831	6.7129	46,840	11.9
Quercitrin	Flavonoid-3-O-glycosides	18.322	447.09387	7.9544	46,688	11.9

Table 3. Cont.

Identified Compounds	Ontology	ART (min)	AMz	Total Score	Peak Height	Conc. (mg/L)
Ethyl 7-epi-12-hydroxyjasmonate glucoside	Fatty acyl glycosides of mono- and disaccharides	18.513	415.19803	6.1113	294	0.1
Kaempferol 3- α -L-arabinopyranoside	Flavonoid glycoside	18.969	417.08301	7.5182	11,122	2.8
Phlorizin	Polyphenol glycoside	19.097	435.1297	7.9116	24,326	6.2

“ART”, average retention time; “AMz”, average mass-to-charge ratio; “Conc.”, concentration.

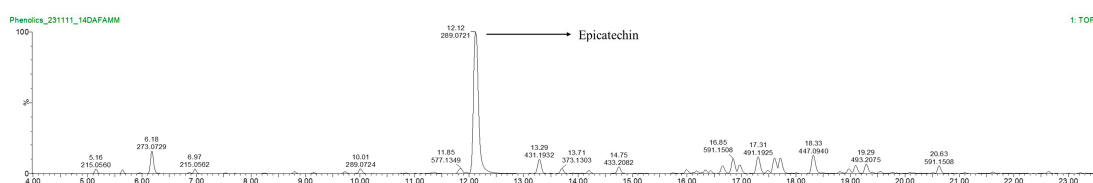


Figure 2. LC-MS chromatogram of fraction 5.

Subjecting fraction 5 to Sephadex chromatography yielded seven sub-fractions, which were assessed for their radical scavenging, anti-lipid peroxidative, α -glucosidase inhibitory and cellular glucose uptake activities. The anti-lipid peroxidative and α -glucosidase inhibitory activities of sub-fractions 4 and 5 significantly ($p < 0.05$) outperform the activities of the other sub-fractions (Table 4). In fact, at a tested concentration of 20 $\mu\text{g/mL}$, their anti-lipid peroxidative and α -glucosidase activities were comparable to that of Trolox and acarbose, respectively. The data suggest that the epicatechin principle in fraction 5 (Table 3 and Figure 2) may have been eluted in sub-fractions 4 and 5, which influenced their potent activities. Moreover, the protective effect of epicatechin and derivatives on lipid peroxidative damage [30,31], as well as their α -glucosidase inhibitory effects [27–29], have been documented.

Table 4. In vitro radical scavenging, anti-linoleic acid peroxidative, enzyme inhibitory and glucose uptake modulatory activities of sub-fractions at 20 $\mu\text{g/mL}$.

Sub-Fractions and Standards	DPPH (%)	ABTS (%)	AnLP (%)	AGI (%)	GUMA (%)
SBF1	37.7 $\pm$ 4.26 ^{cd}	84.8 $\pm$ 3.96 ^{bc}	25.9 $\pm$ 7.00 ^{ab}	22.4 $\pm$ 2.69 ^a	NAD
SBF2	26.7 $\pm$ 6.24 ^{abc}	72.8 $\pm$ 1.34 ^a	19.9 $\pm$ 2.21 ^a	36.4 $\pm$ 4.03 ^{ab}	NAD
SBF3	22.1 $\pm$ 3.81 ^{ab}	72.1 $\pm$ 4.63 ^a	38.7 $\pm$ 5.52 ^b	46.8 $\pm$ 12.7 ^{bc}	14.0 $\pm$ 6.92 ^a
SBF4	32.2 $\pm$ 6.36 ^{bcd}	86.8 $\pm$ 2.54 ^c	65.5 $\pm$ 8.95 ^c	65.8 $\pm$ 2.85 ^d	38.2 $\pm$ 5.81 ^c
SBF5	26.1 $\pm$ 1.99 ^{abc}	87.1 $\pm$ 0.90 ^c	60.1 $\pm$ 3.72 ^c	56.0 $\pm$ 3.00 ^{cd}	28.0 $\pm$ 2.47 ^b
SBF6	18.2 $\pm$ 4.00 ^a	91.8 $\pm$ 4.31 ^c	29.6 $\pm$ 7.80 ^{ab}	29.2 $\pm$ 6.00 ^a	NAD
SBF7	15.3 $\pm$ 4.89 ^a	86.0 $\pm$ 1.71 ^c	23.4 $\pm$ 6.95 ^{ab}	23.9 $\pm$ 5.22 ^a	11.7 $\pm$ 3.28 ^a
Asc	40.0 $\pm$ 2.06 ^d	75.9 $\pm$ 2.77 ^{ab}	ND	N/A	N/A
Trolox	68.2 $\pm$ 3.87 ^e	76.6 $\pm$ 1.83 ^{ab}	70.7 $\pm$ 4.05 ^c	N/A	N/A
Aca	N/A	N/A	N/A	58.8 $\pm$ 4.04 ^{cd}	N/A

“ABTS”, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; “Aca”, acarbose; “AGI”, α -glucosidase inhibitory activity; “AnLP”, anti-linoleic acid peroxidation activity; “Asc”, ascorbic acid; “DPPH”, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; “GUMA”, glucose uptake modulatory activity; “N/A”, not applicable; “ND”, not determined. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the superscript letters represent significant differences ($p < 0.05$) between groups when there are no similar letters.

Furthermore, sub-fractions 4 and 5 show stronger ($p < 0.05$) glucose uptake modulatory activities relative to the other sub-fractions in Chang cells (Table 4). However, sub-fraction 4 is not as potent as sub-fraction 5 ($p < 0.05$). Cellular glucose uptake is a key mechanism

in physiological glucose homeostasis, which is impaired during T2D due to insulin resistance [3]. Thus, the potent glucose uptake modulatory activity of sub-fraction 4 suggests it contains flavonoid(s) that can potentiate glycaemic control via improving insulin signalling and cellular glucose uptake and utilisation. Epicatechin was the most plausible influencing bioactive flavonoids of sub-fraction 4 because it was the most abundant flavonoid in the parent fraction (fraction 5) (Table 3 and Figure 2). It is a dietary supplement reported to improve cellular insulin sensitivity and glucose tolerance in obese and T2D individuals [32]. Hence, sub-fraction 4 was probed further to isolate bioactive epicatechin.

3.3. Spectrometric and Bioactive Profiles of Isolated Epicatechin

Epicatechin was isolated from sub-fraction 4 using preparative TLC as a light/pale yellow powder. The spectral data of epicatechin (Figure 3) is as follows: ESI-MS⁺: m/z 291 [M + H]⁺, 313 [M + Na]⁺ and 387 [M + K]⁺ (Figure S1); ESI-MS[−]: m/z 289 [M − H][−], 325 [M + Cl][−] and 579 [2M − H][−] (Figure S2). ¹H-NMR spectrum (400 MHz, CD₃OD) is as shown in Table 5: δ_H 6.99 (1H, s, H-2'), δ_H 6.80 (1H, dd, J = 8.2 Hz, H-6'), δ_H 6.78 (1H, d, 8.1 Hz, H-5'), δ_H 5.96 (1H, d, H-6), δ_H 5.94 (1H, d, H-8), δ_H 4.84 (1H, brs, H-2), δ_H 4.19 (1H, s, H-3), δ_H 2.88 (1H, dd, J = 4.3, 16.8 Hz, H-4axi) and δ_H 2.75 (1H, dd, H-4eq), see Table 1. ¹³C-NMR (100 MHz, CD₃OD): δ_C 156.6 (C-5), 156.3 (C-8a), 156.0 (C-7), 144.6 (C-3'), 144.4 (C-4'), 130.9 (C-1'), 113.9 (C-2'), 118.0 (C-6'), 114.5 (C-5'), 98.7 (C-4a), 95.0 (C-6), 94.5 (C-8), 78.5 (C-2), 66.1 (C-3) and 27.9 (C-4).

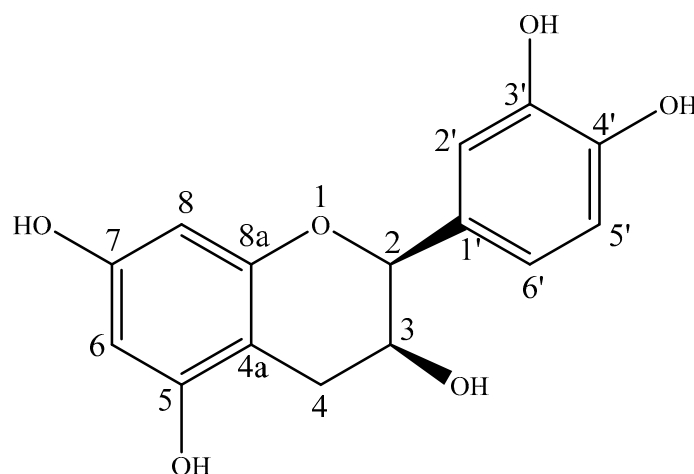


Figure 3. Structure of (+)-Epi-catechin.

The ¹H-NMR (400 MHz) spectrum of the compound showed four groups of chemical shift values attributable to the flavonoid's skeleton [33]. The first group at δ_H 6.99 (1H, s, H-2'), δ_H 6.80 (1H, dd, J = 8.2 Hz, H-6') and δ_H 6.78 (1H, d, 8.1 Hz, H-5') form an ABX system (trisubstituted benzene ring-B of flavonoids); the second group at δ_H 5.96 (1H, d, H-6) and δ_H 5.94 (1H, d, H-8) correspond to an AX system (tetrasubstituted benzene ring-A of flavonoids); the third and fourth groups formed by two oxymethine protons at δ_H 4.84 (1H, brs, H-2) and δ_H 4.19 (1H, s, H-3); and two methylene anisotropic protons at δ_H 2.88 (1H, dd, J = 4.3, 16.8 Hz, H-4axi) and δ_H 2.75 (1H, dd, H-4eq) (Figure S3) are attributable to proton 2, 3 and 4 of saturated ring C of flavan-3-ol type flavonoid, respectively [33,34]. The ¹³C-NMR (100 MHz, CD₃OD) spectrum indicated the presence of 15 carbon atoms [five oxygenated aromatic carbons at δ_C 156.6 (C-5), 156.3 (C-8a), 156.0 (C-7), 144.6 (C-3') and 144.4 (C-4'); five aromatic methine carbons at 113.9 (C-2'), 118.0 (C-6'), 114.5 (C-5'), 95.0 (C-6) and 94.5 (C-8); two aromatic quaternary carbons at 130.9 (C-1') and 98.7 (C-4a); two aliphatic oxygenated methine carbons at 78.5 (C-2) and 66.1 (C-3), and one aliphatic methylene carbon at 27.9 (C-4)] (Figure S4). The DEPT 135 spectrum confirms the presence of one methylene and seven methine carbons. All the above above-mentioned information confirms compound 1 to be a flavan-3-ol flavonoid type [35]. In the HSQC, HMBC, COSY

and NOESY spectra (Figures S5–S8), the structure of the compound was a catechin epimer. The presence of signals of asymmetric carbons around δ 79 (C-2) and 67 (C-3) shows the compound is an epicatechin rather than a catechin [signals around δ 82 (C-2) and 68 (C-3)]. The chemical shift values of protons H-2 and H-3 around δ 4.85 and 4.18 confirms the compound is a (+)-*Epi*-catechin [36], which has been previously isolated from *Euphorbia thymifolia* linn. and characterised [37], as shown in Table 5.

Table 5. ^1H (400 MHz) and ^{13}C (100 MHz) NMR data of the isolated compound compared with reported literature.

Position	Compound 1		Reported Literature [36]	
	δ ^1H (J in Hz)	δ ^{13}C	δ ^1H (J in Hz)	δ ^{13}C
2	4.84 (s)	78.5	4.85 (s)	79.6
3	4.19 (s)	66.1	4.18 (br s)	67.1
4 _{axi}	2.88 (dd, $J = 4.3$; 16.8 Hz)	27.9	2.83 (br d, 17.0 Hz)	29.2
4 _{eq}	2.75 (dd, $J = 4.3$; 16.8 Hz)	27.9	2.70 (br d, 17.0 Hz)	29.2
4 _a		98.7		100.0
5		156.6		157.8
6	5.96 (d)	95.0	5.99 (d, $J = 1.8$ Hz)	96.3
7	5.94 (d)	156.0	5.88 (d, $J = 1.8$ Hz)	157.8
8		94.5		95.5
8 _a		156.3		157.4
1'		130.9		132.5
2'	6.99 (s)	113.9	7.01 (d, 1.8 Hz)	115.5
3'		144.6		145.6
4'		144.4		145.5
5'	6.78 (d, $J = 8.1$ Hz)	114.5	6.75 (d, $J = 8.1$ Hz)	115.7
6'	6.80 (dd, $J = 8.1$; 2.2 Hz)	118.0	6.80 (dd, $J = 8.0$; 1.8 Hz)	119.6

Bioactivity assessment of the isolated epicatechin shows that it demonstrates a dose-dependent inhibitory action ($\text{IC}_{50} = 18.9 \mu\text{M}$) on LPS-induced ROS production in RAW 264.7 macrophages, which is statistically comparable ($p > 0.05$) to that of ascorbic acid ($\text{IC}_{50} = 9.57 \mu\text{M}$) (Table 6 and Figure 4), reiterating the antioxidant potency of epicatechin. LPS-induced ROS production has been linked to proinflammatory signalling, which can disrupt cellular processes and cause oxidative tissue injuries due to the excessive production of superoxide ions and hydrogen peroxide [38]. Hence, it is plausible that the inhibitory effect of epicatechin on LPS-induced ROS production in RAW 264.7 macrophages was linked to both antioxidant and anti-inflammatory capacities. Moreover, the inhibitory effect of epicatechin on LPS-induced production of proinflammatory mediators and cytokines in RAW 264.7 macrophages has been reported [39].

Table 6. IC_{50} and EC_{50} values for the dose-dependent activities of the tested samples.

Assays	Epicatechin	Acarbose	Ascorbic Acid
	IC_{50} and EC_{50} Values (μM)		
α -Glucosidase inhibitory activity (IC_{50})	35.3 ± 5.75^a	11.0 ± 4.11^b	NA
Inhibition of ROS production in RAW 264.7 cells (IC_{50})	18.9 ± 5.44	NA	9.57 ± 4.10
Chang cell glucose uptake modulatory activity (EC_{50})	78.5 ± 13.6	NA	NA

“NA” means “not applicable”. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the superscript letters attached to each dataset represent significant differences ($p < 0.05$) between groups when there are no similar letters.

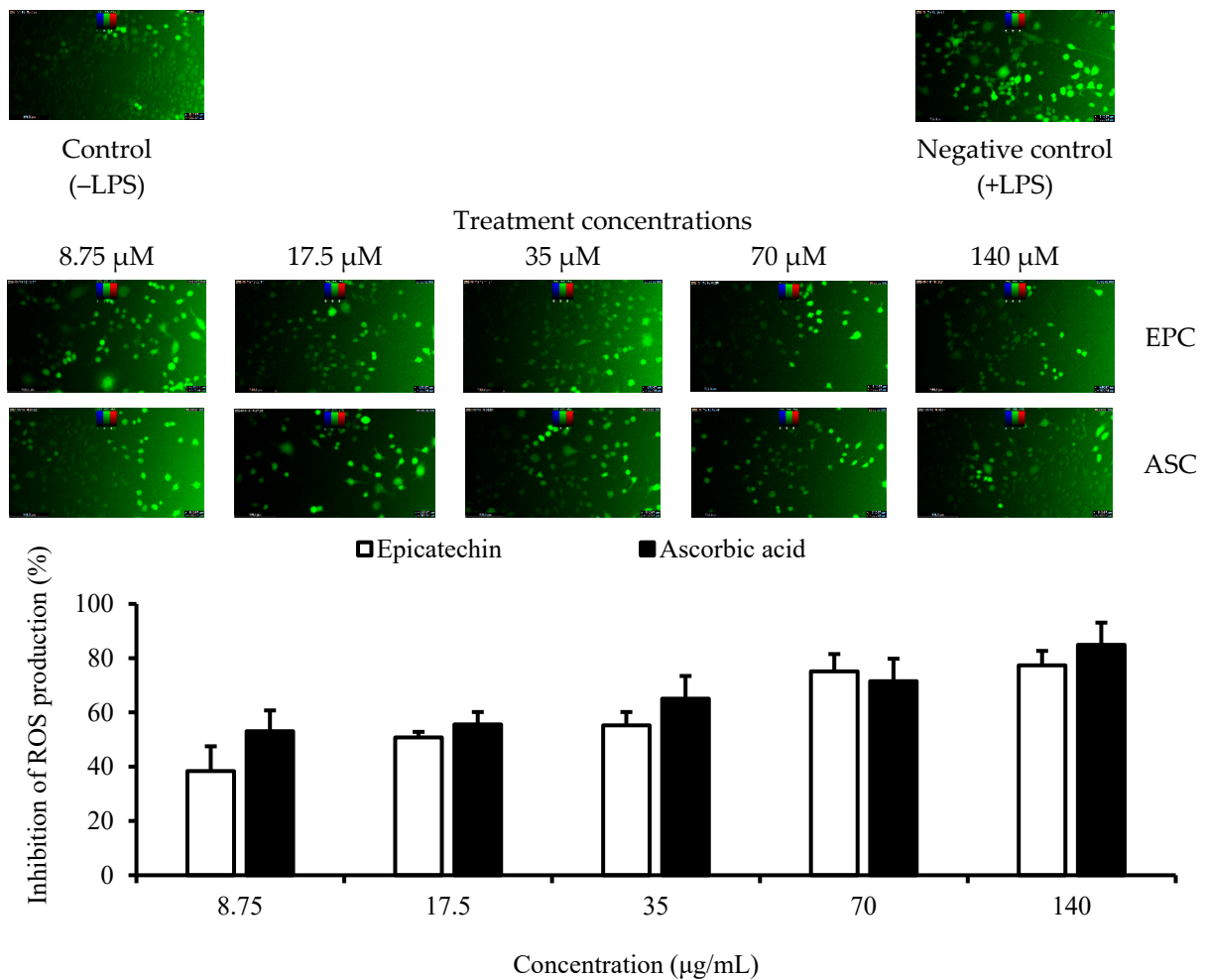


Figure 4. Dose-dependent inhibitory effect of epicatechin (EPC) and ascorbic acid (ASC) on ROS production in LPS-treated RAW 264.7 macrophages. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups at a given concentration when there are no similar letters. “LPS” means lipopolysaccharide.

Furthermore, the isolated epicatechin demonstrated a dose-dependent glycaemic control potential. It has an inhibitory effect ($\text{IC}_{50} = 35.3 \mu\text{M}$) on α -glucosidase (Table 6 and Figure 5), suggesting it could be a key flavonoid influencing the α -glucosidase inhibitory activity of the potent sub-fraction(s)/fraction(s) and extract of litchi fruit peel. In Chang cells, epicatechin exerts a dose-dependent modulatory effect ($\text{EC}_{50} = 78.5 \mu\text{M}$) on glucose uptake (Table 6 and Figure 6).

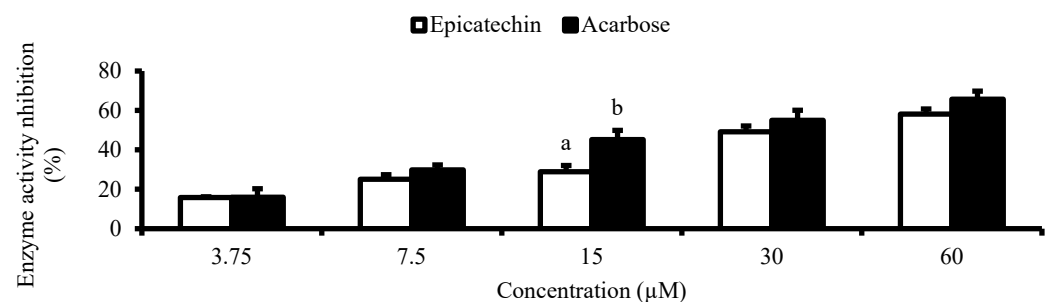


Figure 5. Dose-dependent inhibitory effect of epicatechin and acarbose on α -glucosidase activity. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups at a given concentration when there are no similar letters.

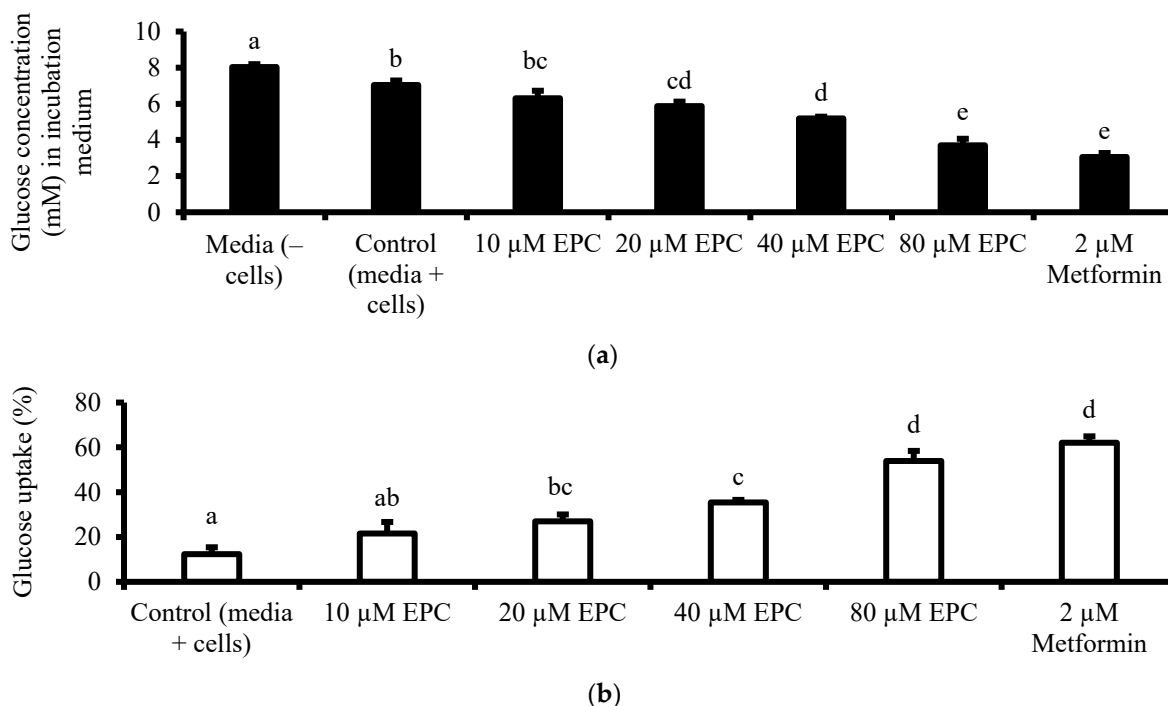


Figure 6. (a) Glucose concentration in the medium of cells receiving different treatments and (b) % glucose uptake of cells receiving different treatments. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between the treatments when there are no similar letters. “EPC” means epicatechin.

Relative to the untreated cells (control), an 80 μ M epicatechin concentration enhances ($p < 0.05$) glucose uptake to an extent that is statistically comparable ($p > 0.05$) to the glucose uptake effect of a 2 μ M Metformin, suggesting epicatechin may be effective in promoting cellular glucose uptake in cells of peripheral tissues, and may have influenced the glucose uptake activity of the potent sub-fractions 4 and 5 (Table 4). Documented evidence suggests that epicatechin may enhance glucose uptake by modulating insulin signalling via the PI3K/AKT signalling pathway and modulating the translocation of glucose transporters [28,40]. Further studies are, however, needed to investigate this speculation.

4. Conclusions

In the present study, a bioassay-guided approach was used to probe into litchi fruit peel’s antioxidant and anti-hyperglycaemic phytochemistry. Ethyl acetate solvent effectively extracted key flavonoids, which potentiated more potent antioxidant and enzyme inhibitory activities relative to the other solvent extraction. The flavonoid contents of fractions from the ethyl acetate extract also correlated with their α -glucosidase inhibitory activities. Epicatechin was the most abundant flavonoid in the fraction (fraction 5), with the highest inhibitory activity. Epicatechin was isolated from a sub-fraction (sub-fraction 4) of fraction 5. The isolated epicatechin demonstrated a dose-dependent inhibitory effect on α -glucosidase, modulatory effect on cellular glucose uptake, inhibitory effect on cellular ROS production, and possibly influenced the potent α -glucosidase inhibitory, glucose uptake modulatory and anti-lipid peroxidative activity demonstrated by sub-fraction 4. The data of this study showed that litchi peel EtOAc extract may exert antioxidant and glycaemic control by alleviating ROS-mediated oxidative insults, inhibiting carbohydrate digesting enzymes and modulating cell glucose uptake, and epicatechin may be a principal bioactive flavonoid influencing these antioxidant and glycaemic control properties. Thus, litchi peel EtOAc extract can be an alternative source to recover epicatechin with cellular glucose uptake modulatory and ROS production suppressive capacities.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox13101233/s1>, Figure S1: ESIMS (+) spectrum of the isolated compound; Figure S2: ESIMS (-) spectrum of the isolated compound; Figure S3: ^1H NMR (400 MHz, CD_3OD) spectrum of the isolated compound; Figure S4: ^{13}C NMR (100 MHz, CD_3OD) spectrum of the isolated compound; Figure S5: HSQC spectrum (CD_3OD) of the isolated compound; Figure S6: HMBC spectrum (CD_3OD) of the isolated compound; Figure S7: COSY spectrum (CD_3OD) of the isolated compound; Figure S8: NOESY spectrum (CD_3OD) of the isolated compound; Table S1: Non-polar to polar eluent system used gradient column fractionation of the EtAc extract; Table S2: Yield of fractions from gradient column chromatography of the EtAc extract.

Author Contributions: Methodology, G.O.I., N.P.M., J.N., M.S.C. and E.M.N.; formal analysis, G.O.I., N.P.M., J.N., M.S.C., E.M.N., G.T.T., S.L.B., T.J.M. and C.I.C.; investigation, G.O.I., N.P.M. and J.N.; data curation, G.O.I., N.P.M., J.N., M.S.C., E.M.N., G.T.T. and S.L.B.; writing—original draft, G.O.I.; conceptualization, C.I.C.; resources, T.J.M., S.S.M. and C.I.C.; supervision, S.S.M. and C.I.C.; funding acquisition, C.I.C.; writing—review and editing, G.O.I., N.P.M., J.N., M.S.C., E.M.N., G.T.T., S.L.B., T.J.M., S.S.M. and C.I.C. All authors have read and agreed to the published version of the manuscript.

Funding: The study was funded by the postgraduate scholarship (reference: MND200429517886) and incentive funding for rated researchers (grant No: 150945) of the National Research Foundation, South Africa.

Institutional Review Board Statement: The protocol reported in this study was approved by the Faculty Research and Innovation Committee of the Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein, on 18 May 2021.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. International Diabetes Federation. *IDF Diabetes Atlas*, 10th ed.; IDF: Brussels, Belgium, 2021; Available online: https://diabetesatlas.org/idfawp/resource-files/2021/07/IDF_Atlas_10th_Edition_2021.pdf (accessed on 2 January 2022).
2. Zhu, J.; Chen, M.; Pang, Y.; Li, S. Impact of lifestyle education for type 2 diabetes mellitus: Protocol for a randomized controlled trial. *J. Med.* **2021**, *100*, e24208. [CrossRef] [PubMed]
3. Galicia-Garcia, U.; Benito-Vicente, A.; Jebari, S.; Larrea-Sebal, A.; Siddiqi, H.; Uribe, K.B.; Ostolaza, H.; Martín, C. Pathophysiology of Type 2 Diabetes Mellitus. *Int. J. Mol. Sci.* **2020**, *21*, 6275. [CrossRef] [PubMed]
4. Bhatti, J.S.; Sehrawat, A.; Mishra, J.; Sidhu, I.S.; Navik, U.; Khullar, N.; Kumar, S.; Bhatti, G.K.; Reddy, P.H. Oxidative stress in the pathophysiology of type 2 diabetes and related complications: Current therapeutics strategies and future perspectives. *Free Radic. Biol. Med.* **2022**, *184*, 114–134. [CrossRef] [PubMed]
5. Sun, C.; Zhao, C.; Guven, E.; Paoli, P.; Simal-Gándara, J.; Ramkumar, K.M.; Wang, S.; Buleu, F.N.; Pah, A.M.; Turi, V.; et al. Dietary polyphenols as antidiabetic agents: Advances and opportunities. *Food Front.* **2020**, *1*, 18–44. [CrossRef]
6. Afam, I.O.J.; Silungwe, H.; Takalani, T.; Omolola, A.O.; Udeh, H.O.; Anyasi, T.A. Antioxidant-rich natural fruit and vegetable products and human health. *Int. J. Food Prop.* **2021**, *24*, 41–67. [CrossRef]
7. Zhao, L.; Wang, K.; Wang, K.; Zhu, J.; Hu, Z. Nutrient components, health benefits, and safety of litchi (*Litchi chinensis* Sonn.): A review. *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 2139–2163. [CrossRef]
8. Cano-Gómez, C.I.; Alonso-Castro, A.J.; Carranza-Alvarez, C.; Wong-Paz, J.E. Advancements in *Litchi chinensis* Peel Processing: A Scientific Review of Drying, Extraction, and Isolation of Its Bioactive Compounds. *Foods* **2024**, *13*, 1461. [CrossRef]
9. Chukwuma, C.I.; Izu, G.O.; Chukwuma, M.S.; Samson, M.S.; Makhafola, T.J.; Erukainure, O.L. A review on the medicinal potential, toxicology, and phytochemistry of litchi fruit peel and seed. *J. Food Biochem.* **2021**, *45*, e13997. [CrossRef] [PubMed]
10. Tanaka, M.R.R.; Mataruco, L.d.S.; Frota, E.G.; Coelho, B.E.S.; Lima, M.d.S.; Pimentel, T.C.; Barão, C.E. Lychee peel extract obtained by ultrasound-assisted extraction: Bioactive compounds and functional properties. *Acta Sci. Technol.* **2024**, *46*, e66447. [CrossRef]
11. Rong, S.; Hu, X.; Zhao, S.; Zhao, Y.; Xiao, X.; Bao, W.; Liu, L. Procyanidins extracted from the litchi pericarp ameliorate atherosclerosis in ApoE knockout mice: Their effects on nitric oxide bioavailability and oxidative stress. *Food Funct.* **2017**, *8*, 4210–4216. [CrossRef]
12. Queiroz, E.R.; Abreu, C.; Rocha, D.A.; Sousa, R.V.; Fráguas, R.M.; Braga, M.A.; César, P. Lychee (*Litchi chinensis* Sonn.) peel flour: Effects on hepatoprotection and dyslipidemia induced by a hypercholesterolemic diet. *An. Acad. Bras. Ciênc.* **2018**, *90*, 267–281. [CrossRef] [PubMed]

13. Yang, Z.; Zhang, L.; Liu, J.; Li, D. Litchi Pericarp Extract Treats Type 2 Diabetes Mellitus by Regulating Oxidative Stress, Inflammatory Response, and Energy Metabolism. *Antioxidants*. **2024**, *13*, 495. [[CrossRef](#)] [[PubMed](#)]
14. Zhao, M.; Yang, B.; Wang, J.; Li, B.; Jiang, Y. Identification of the major flavonoids from pericarp tissues of lychee fruit in relation to their antioxidant activities. *Food Chem.* **2006**, *98*, 539–544. [[CrossRef](#)]
15. Zhao, M.; Yang, B.; Wang, J.; Liu, Y.; Yu, L.; Jiang, Y. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn) pericarp. *Int. Immunopharmacol.* **2007**, *7*, 162–166. [[CrossRef](#)]
16. Li, J.; Jiang, Y. Litchi Flavonoids: Isolation, Identification and Biological Activity. *Molecules* **2007**, *12*, 745–758. [[CrossRef](#)]
17. Prasad, K.N.; Chew, L.Y.; Khoo, H.E.; Kong, K.W.; Azlan, A.; Ismail, A. Antioxidant capacities of peel, pulp, and seed fractions of *Canarium odontophyllum* Miq. fruit. *J. Biomed. Biotechnol.* **2010**, *2010*, 871379. [[CrossRef](#)]
18. Chauhan, S.; Gupta, S.; Yasmin, S.; Saini, M. Antihyperglycemic and Antioxidant Potential of Plant Extract of *Litchi chinensis* and *Glycine max*. *Int. J. Nutr. Pharmacol. Neurol. Dis.* **2021**, *11*, 225–233. [[CrossRef](#)]
19. Chukwuma, C.I.; Mashele, S.S.; Akuru, E.A. Evaluation of the in vitro α -amylase inhibitory, antiglycation, and antioxidant properties of *Punica granatum* L. (pomegranate) fruit peel acetone extract and its effect on glucose uptake and oxidative stress in hepatocytes. *Food Biochem.* **2020**, *44*, e13175. [[CrossRef](#)]
20. Motloun, D.M.; Mashele, S.S.; Matowane, G.R.; Swain, S.S.; Bonnet, S.L.; Noreljaleel, A.E.M.; Oyedemi, S.O.; Chukwuma, C.I. Synthesis, characterization, antidiabetic and antioxidative evaluation of a novel Zn(II)-gallic acid complex with multi-facet activity. *J. Pharm. Pharmacol.* **2020**, *72*, 1412–1426. [[CrossRef](#)]
21. Mashile, B.; Setlhodi, R.; Izu, G.O.; Erukainure, O.L.; Mashele, S.S.; Makhafa, T.J.; Eze, K.C.; Chukwuma, C.I. Temperature-dependent extraction and chromatographic recovery and characterization of ellagitannins with potent antioxidant and glycaemic control properties from “Wonderful” pomegranate peel. *Int. J. Food Sci. Technol.* **2024**, *59*, 408–424. [[CrossRef](#)]
22. Izu, G.O.; Mfotie Njoya, E.; Tabakam, G.T.; Namboozee, J.; Otukile, K.P.; Tsoeu, S.E.; Fasiku, V.O.; Adegoke, A.M.; Erukainure, O.L.; Mashele, S.S.; et al. Unravelling the Influence of Chlorogenic Acid on the Antioxidant Phytochemistry of Avocado (*Persea americana* Mill.) Fruit Peel. *Antioxidants* **2024**, *13*, 456. [[CrossRef](#)] [[PubMed](#)]
23. Chen, J.; Yang, J.; Ma, L.; Li, J.; Shahzad, N.; Kim, C.K. Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Sci. Rep.* **2020**, *10*, 2611. [[CrossRef](#)]
24. Scarano, A.; Laddomada, B.; Blando, F.; De Santis, S.; Verna, G.; Chieppa, M.; Santino, A. The Chelating Ability of Plant Polyphenols Can Affect Iron Homeostasis and Gut Microbiota. *Antioxidants* **2023**, *12*, 630. [[CrossRef](#)] [[PubMed](#)]
25. Dirir, A.M.; Daou, M.; Yousef, A.F.; Yousef, L.F. A review of alpha-glucosidase inhibitors from plants as potential candidates for the treatment of type-2 diabetes. *Phytochem. Rev. Proc. Phytochem. Lett.* **2022**, *21*, 1049–1079. [[CrossRef](#)] [[PubMed](#)]
26. Kashtoh, H.; Baek, K.-H. Recent Updates on Phytoconstituent Alpha-Glucosidase Inhibitors: An Approach towards the Treatment of Type Two Diabetes. *Plants* **2022**, *11*, 2722. [[CrossRef](#)] [[PubMed](#)]
27. Wu, X.; Hu, M.; Hu, X.; Ding, H.; Gong, D.; Zhang, G. Inhibitory mechanism of epicatechin gallate on α -amylase and α -glucosidase and its combinational effect with acarbose or epigallocatechin gallate. *J. Mol. Liq.* **2019**, *290*, 111202. [[CrossRef](#)]
28. Xu, L.; Li, W.; Chen, Z.; Guo, Q.; Wang, C.; Santhanam, R.K.; Chen, H. Inhibitory effect of epigallocatechin-3-O-gallate on α -glucosidase and its hypoglycemic effect via targeting PI3K/AKT signaling pathway in L6 skeletal muscle cells. *Int. J. Biol. Macromol.* **2019**, *125*, 605–611. [[CrossRef](#)]
29. Şöhretöğlu, D.; Renda, G.; Arroo, R.; Xiao, J.; Sari, S. Advances in the natural α -glucosidase inhibitors. *eFood* **2023**, *4*, e112. [[CrossRef](#)]
30. Terao, J.; Piskula, M.; Yao, Q. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch. Biochem.* **1994**, *308*, 278–284. [[CrossRef](#)]
31. Shaki, F.; Shayeste, Y.; Karami, M.; Akbari, E.; Rezaei, M.; Ataee, R. The effect of epicatechin on oxidative stress and mitochondrial damage induced by homocysteine using isolated rat hippocampus mitochondria. *Pharm. Sci. Res.* **2017**, *12*, 119–127. [[CrossRef](#)]
32. Cremonini, E.; Fraga, C.G.; Oteiza, P.I. (–)-Epicatechin in the control of glucose homeostasis: Involvement of redox-regulated mechanisms. *Free Radic. Biol. Med.* **2019**, *130*, 478–488. [[CrossRef](#)] [[PubMed](#)]
33. Tajuddeen, N.; Sallau, M.S.; Musa, A.M.; Yahaya, S.M.; Habila, J.D.; Ismail, A.M. A novel antimicrobial flavonoid from the stem bark of *Commiphora pedunculata* (Kotschy & Peyr.) Engl. *Nat. Prod. Res.* **2016**, *30*, 1109–1115. [[CrossRef](#)] [[PubMed](#)]
34. Jung, E.K.; Sang, S.K.; Chang-Gu, H.; Nam, H.L. Antioxidant Chemical Constituents from the Stems of *Cleyera japonica* Thunberg. *Int. J. Pharmacol.* **2012**, *8*, 410–415. [[CrossRef](#)]
35. Abdullahi, S.M.; Musa, A.M.; Abdullahi, M.I.; Sani, Y.M.; Atiku, I. Catechin from the leaf extract of *Ziziphus mucronata* Willd. (Rhamnaceae). *Niger. J. Pharm. Sci.* **2017**, *16*, 1–5.
36. Abd El-Razek, M.H. NMR Assignments of Four Catechin Epimers. *Asian J. Chem.* **2007**, *19*, 4867–4872.
37. Kainsa, S.; Singh, R. Flavan-3-ol Isomers Isolated from *Euphorbia Thymifolia* Linn. *Pharmacogn. Commun.* **2016**, *6*, 28–33. [[CrossRef](#)]
38. Hsu, H.Y.; Wen, M.H. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.* **2002**, *277*, 22131–22139. [[CrossRef](#)]

39. Wang, H.; Cao, Z. Anti-inflammatory Effects of (–)-Epicatechin in Lipopolysaccharide-Stimulated Raw 264.7 Macrophages. *Trop. J. Med. Res.* **2014**, *13*, 1415–1419. [[CrossRef](#)]
40. Chun, J.H.; Henckel, M.M.; Knaub, L.A.; Hull, S.E.; Pott, G.B.; Ramirez, D.G.; Reusch, J.E.; Keller, A.C. (–)-Epicatechin Reverses Glucose Intolerance in Rats Housed at Thermoneutrality. *Planta Medica* **2022**, *88*, 735–744. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.



Article

Investigating the Phytochemistry and Underlying Glycemic Control Mechanisms of *Litchi chinensis* Sonn. (Litchi) Peel Ethyl Acetate Extract in a Fructose/Streptozotocin Diabetic Model of Rats

Gloria O. Izu ^{1,2} , Samson S. Mashele ² and Chika I. Chukwuma ^{2,*} ¹ Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein 9301, Free State, South Africa; 221039621@stud.cut.ac.za² Centre for Quality of Health and Living (CQHL), Faculty of Health and Environmental Sciences, Central University of Technology, Private Bag X20539, Bloemfontein 9300, Free State, South Africa; smashele@cut.ac.za

* Correspondence: chykochi@yahoo.com or cchukwuma@cut.ac.za; Tel.: +27-(0)-51-507-4028

Abstract: The glycemic control potential and flavonoid profile of litchi have been documented for its hydroalcoholic extracts, while there is scarce information regarding its ethyl acetate extract. This study investigated the flavonoid profile, as well as the ameliorative potential and possible underlying mechanisms of litchi peel ethyl acetate extract on type 2 diabetes-related pathologies in a fructose/streptozotocin (STZ) model of diabetic rats. Sprague Dawley rats were induced with diabetes by administering 10% fructose for 2 weeks and a single i.p. injection of low-dose (40 mg/kg bw) STZ. Thereafter, the animals were orally administered with a low-dose (150 mg/kg bw) and high-dose (300 mg/kg bw) of the peel extract (LDPE and HDPE, respectively) and metformin (200 mg/kg bw). Compared to untreated diabetic rats (AUC = 1004 mg.h/dL), the HDPE significantly ($p < 0.05$) improved glucose tolerance (AUC = 847 mg.h/dL), which was statistically comparable ($p > 0.05$) to the effect of metformin (AUC = 903 mg.h/dL). Serum insulin and pancreatic histology data showed that the STZ-induced pancreatic damage and insulin depletion was improved by the HDPE, which could be linked to the observed ameliorative effect of the extract on pancreatic lipid peroxidation and SOD and catalase activity. The extract further improved liver and muscle glycogen storage, as well as muscle hexokinase activity and Akt phosphorylation, suggesting that the extract exerts glycemic control by enhancing glycogen storage and modulating insulin-mediated signaling of glucose uptake and utilization. LC-MS data and documented reports suggest that flavonoids, such as epicatechin, cinnamtannin B2, procyanidin B5, and proanthocyanidin A2, are the possible influencing compounds. The ethyl acetate extract of litchi peel could be a source of bioactive flavonoids that can potentiate glycemic control in diabetes and mitigate oxidative stress-related pathologies.



Citation: Izu, G.O.; Mashele, S.S.; Chukwuma, C.I. Investigating the Phytochemistry and Underlying Glycemic Control Mechanisms of *Litchi chinensis* Sonn. (Litchi) Peel Ethyl Acetate Extract in a Fructose/Streptozotocin Diabetic Model of Rats. *Nutrients* **2024**, *16*, 3644. <https://doi.org/10.3390/nu16213644>

Academic Editor: Lindsay Brown

Received: 28 September 2024

Revised: 19 October 2024

Accepted: 22 October 2024

Published: 26 October 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Keywords: antioxidant; ethyl acetate extract; flavonoids; fructose/streptozotocin diabetic rats; glycemic control; litchi fruit peel

1. Introduction

According to the International Diabetes Federation [1], the global prevalence of adults suffering from diabetes will increase from 537 million in 2021 to 783 million in 2045 [1], suggesting that the negative impact of diabetes on global health and socioeconomic landscapes could worsen if precautionary measures are not implemented. Diabetes is characterized by detrimental metabolic imbalances caused by hormonal insufficiency or dysfunction, which results in persistent hyperglycemia [2]. Type 2 diabetes (T2D) is the most prevalent type of diabetes, possibly due to its association with lifestyle-related factors, such as physical inactivity, dieting, and detrimental weight gain [3]. These factors lead to insulin resistance, which is a key pathophysiological defect of T2D [3,4]. During insulin resistance,

the homeostatic regulatory action of insulin on circulating glucose is impaired due to dysfunctional signaling in the cells of peripheral tissues [4]. Cellular glucose uptake, nutrient metabolism, and utilization are impaired, which leads to reduced glycemic control and eventually persistent hyperglycemia, especially if the function of the pancreatic β -cells has been concomitantly compromised [4].

Persistent hyperglycemia has been linked to the development of microvascular and macrovascular complications of diabetes and oxidative stress has been documented as a key underlying mediator [5]. Oxidative stress is caused by an imbalance between the physiological production of deleterious prooxidants or reactive oxygen species (ROS) and the antioxidant mechanisms to mop or quench the oxidant action of the prooxidants [6]. During diabetes, hyperglycemia facilitates the production of ROS, which can oxidatively damage cellular macromolecules and organelles, while signaling associated inflammatory responses [5]. High blood glucose can cause glycation to key functional macromolecules, leading to the production of advanced end products (AGEs), which are biological indicators of degenerative diseases, including diabetic complications [5]. Elevated lipid peroxidation in diabetes has also been documented, which can compromise cellular integrity due to damage to the lipid bilayer of cell membranes [5]. Also, deleterious radical byproducts of lipid peroxidation can increase oxidative stress and cause oxidative damage to macromolecules [5].

While there are many available commercial antidiabetic drugs, medicinal plants, including fruits and vegetables are increasingly been used as complementary and/or alternative therapies. Dietary antioxidant phenolic compounds present in fruits and vegetables offer a broad spectrum of pharmacological activities that could mitigate the development of diabetic complications by suppressing oxidative stress and inflammatory responses [7]. *Litchi chinensis* Sonn. (Sapindaceae) is a tropical and subtropical fruit with high commercial value due to its global consumption [8]. The nutritional value and medicinal benefits of the fruit have also been documented [8]. While studies have mentioned the antioxidant, anti-hyperglycemic, anti-hypertensive, anti-obesogenic, anti-dyslipidaemic, and hepatoprotective potentials of the fruit [8], documented evidence has shown that the non-edible peel could also be of medicinal relevance [9,10]. Flavonoids, including catechins, procyanidins, proanthocyanidins, quercetin, rutin, and their derivatives have been reported in the peel, which have been linked to some of the reported diabetes-related and antioxidant pharmacological properties of the peel [9–11]. It has been shown that the fruit peel has a strong α -glucosidase inhibitory potential [12], indicating it could potentiate glycemic control by reducing postprandial blood glucose increase [13]. In high-fat-diet-fed rodents, the peel powder and its procyanidin-rich fraction were shown to improve the profiles of blood lipids and glucose, thus ameliorating diabetes-related metabolic alterations [14,15]. In *db/db* mice, litchi peel ethanol extract treatment has been shown to ameliorate type 2 diabetic pathologies, including elevated blood glucose, hepatic gluconeogenesis, oxidative stress, and associated inflammatory responses [16].

A review of the pharmacology potential of litchi fruit seed and peel [9] suggests that diabetes-related pharmacological investigations have mostly been demonstrated in the hydro-alcoholic extracts or isolates with minimal investigations on other solvent extracts, such as the ethyl acetate extract. Ethyl acetate solvent extraction can cause hydrogen bond formation with carboxyl and hydroxyl groups, resulting in the extraction of medium polar to polar phenolics and/or flavonoids [17], which could influence potent medicinal properties. In fact, documented qualitative evidence has shown that the ethyl acetate extract of litchi peel contains a relatively similar proportion of phenolic compounds and flavonoids as the hydroalcoholic extract [18]. A previous study has proven that litchi fruit ethyl acetate extract demonstrates potent immunomodulatory potential [19]. In a more recent antidiabetic study, a comparative assessment in alloxan-induced type 1 diabetic Wistar rats suggests that the blood glucose lowering, and antioxidant effect of litchi peel ethyl acetate extract outperformed that of the hydroalcoholic extract [18], suggesting that litchi peel ethyl extract may possess ameliorative potential against T2D-associated pathologies.

However, this speculation, as well as the possible mechanisms, remain elusive and warrants investigation. Therefore, this study investigated the ameliorative potential and possible underlying mechanisms of litchi peel ethyl acetate extract on T2D-related pathologies in a fructose/low-dose streptozotocin (STZ) model of diabetic rats.

2. Materials and Methods

2.1. Plant Material and Extraction

Litchi fruit (McLean's Red cultivar) was purchased from a local fruit and vegetable store in Bloemfontein, South Africa. Clean water was used to wash the fruits, and the peel of the fruits was collected. An oven set at 30 °C was used to dry the peel of the fruits (Model No. 072160, Prolab instrument, Sep Sci., Johannesburg, South Africa). The dried peel was ground. About 300 g of the ground peel was defatted with 1.5 L of hexane. In order to increase polarity, sequentially extraction was conducted on the defatted peel using 1.5 L of dichloromethane (DCM) and ethyl acetate (EtAc) solvents. The extraction was conducted for 48 h at room temperature on an orbital shaker [OrbiShake, Model 262, Labotec 106 (Pty) Ltd., Johannesburg, South Africa] set at 125 rpm. The concentrated ethyl acetate extract was recovered using a Buchi 108 Rotavapor® R-300 [Labotec (Pty) Ltd., Johannesburg, South Africa] and dried under a fume hood. The yield of the extract was determined, before storing it in clean screw-cap vials at −20 °C.

2.2. LC-MS Analysis of the Ethyl Acetate Extract of Litchi Peel

To perform the LC-MS protocol, the following were used: A Waters Synapt G2 (Waters Corporation, Milford, MA, USA), an ESI probe, and an ESI Pos, at a 15 V cone voltage. The operation of liquid chromatography was conducted using an Acquity binary solvent manager. The chromatographic column used was a HSS T3 (Waters Corporation, Milford, MA, USA). It had a dimension of 2.1 × 150 mm and a particle size of 1.8 µm. Two solvent systems (A and B) were used as the mobile phase. Solvent system A was water containing 0.1% methanoic acid, while solvent system B was acetonitrile containing 0.1% methanoic acid. A 0.25 mL/min flow rate was applied for the gradient chromatographic separation at 30 °C. The gradient condition followed the following sequence: 100% (A):0% (B), 10 min; 72% (A):28% (B), 21 min; 60% (A):40% (B), 50 s; 0% (A):100% (B), 2 min. The extract was dissolved in a mixture of acetonitrile and water (1:1 v/v), passed through a 0.22 µm filter, and injected at a volume of 20 µL. The signals of the separated compounds were recorded at 254 nm. Using the accurate masses, the compounds in the fraction were tentatively determined and semi-quantified (mg/L) relative to the epicatechin standard and according to the extracted ions.

2.3. Animals

The approval for the use of animals in this study was obtained from the University of Free State Animal Research Ethics Committee (EREC) on 07 February 2023 (Protocol reference number: UFS-AED2022/0028/23). Thirty-five 7 to 8-week-old Sprague-Dawley (SD) rats were procured by the University of the Free State Animal Research Unit, where the animal experiment was conducted. The animals were allowed to acclimatize for 5 days. Thereafter, the animals were assigned into five groups containing seven animals each. The groups were named the normal control (NC), diabetic control (DBC), low-dose peel extract (LDPE), high-dose peel extract (HDPE), and metformin (MET) groups. The animals were housed two to three animals per cage in large-sized cages with the following dimensions: 598 mm (L) × 380 mm (W) × 200 mm (H) with Grid top hatch access of 220 mm (L) × 225 mm (W) × 70 mm (H). The animals were housed in a temperature (20–24 °C; set point at 22 °C) and humidity (40–70%; set point 60%) controlled room with a 12 h light-dark cycle.

2.4. Diabetes Induction

A previous study [20] has shown that the combination of 10% fructose and low dose (40 mg/kg bw) STZ could be used to develop a diabetic SD rat model that exhibits two

key pathologies of T2D, namely insulin resistance and β -cell damage. This approach was adopted to induce diabetes. First, the rats in the diabetic groups (DBC, LDPE, HDPE, and MET groups) were fed ad libitum with 10% fructose solution in place of drinking water for 2 weeks. After that, the rats were injected with a single intraperitoneal (i.p.) injection of STZ (40 mg/kg bw) dissolved in citrate buffer (pH, 4.4), whereas the rats in the NC group were injected with an equivalent volume of citrate buffer. One week after the STZ injection, the tail-tip blood glucose (mmol/L) of rats was measured using a Contour Plus Blood Glucose Monitoring System (Ascensia Diabetes Care Holdings AG, Basel, Switzerland). A multiplication factor of 18.018 was used to convert the blood glucose measurements from mmol/L to mg/dL. Animals with blood glucose values ranging from 250 to 600 mg/dL were considered diabetic and included in the study. Of the 28 rats induced with diabetes, four rats were extremely diabetic (blood glucose > 600 mg/dL) and thus were excluded from the study. Therefore, each of the diabetic groups was left with six animals each. The number of animals in the NC group was also reduced to six to be consistent with other groups.

2.5. Treatment

After diabetes confirmation, the rats in the different groups were administered via oral gavage with the following treatments:

- NC group: The vehicle of the extract or metformin (5% DMSO solution);
- DBC group: The vehicle of the extract or metformin (5% DMSO solution);
- LDPE: 150 mg/kg bw of extract dissolved in 5% DMSO solution;
- HDPE: 300 mg/kg bw of extract dissolved in 5% DMSO solution;
- MET: 200 mg/kg bw of Metformin hydrochloride (Merck, Johannesburg South Africa) dissolved in dissolved in 5% DMSO solution.

The animals were treated for 20 days during a 4-week intervention period. This implies that the animals were treated during the first five days of each week of the intervention period.

2.6. Measurement of Fluid Intake, Food Intake, and Non-Fasting Blood Glucose

During the intervention period, the average daily fluid intake and average weekly food intake of the animals were measured. The tail-tip non-fasting blood glucose (NFBG) level of the animals was measured on the 6th day of the 1st, 2nd, and 4th weeks of the intervention period. The NFBG was measured using a glucometer.

2.7. Measurement of Oral Glucose Tolerance

The oral glucose tolerance test (OGTT) was performed on the 6th day in the 3rd week of the intervention period. The rats were fasted for 7 h (from 3:00 a.m. to 10:00 a.m.). Thereafter, the tail-tip blood glucose of the animals was measured (0 min) before orally administering 2 g/kg bw of glucose. The tail-tip blood glucose of the animals was then measured for 120 min at 30 min intervals. Thereafter, the animals were allowed access to food. The area under the curve (AUC) for the OGTT was calculated according to a previous report [21]:

$$AUC \text{ (mg}\cdot\text{h/dL)} = \frac{BG_0 + (BG_{30} \times 2) + (BG_{60} \times 3) + (BG_{120} \times 2)}{4}$$

where BG₀, BG₃₀, BG₆₀, and BG₁₂₀ are the mean blood glucose (mg/dL) at 0, 30, 60 and 120 min, respectively.

2.8. Euthanization and Sampling

After the intervention period, the animals were euthanized using isoflurane. The blood of the animals was collected by cardiac puncture and stored in tubes without an anti-coagulant. Also, the liver, pancreas and muscle tissues of the animals were collected. The

collected tissue samples were immediately frozen in liquid and stored at -80°C . A portion of the pancreatic tissues was fixed in 10% neutral buffered formalin (Merck, Johannesburg, South Africa) for histological assessment. The blood was centrifuged at $3500\times g$ for 10 min at 4°C using a NUVE NF 800R centrifuge (Separations, Johannesburg, South Africa) and the supernatant (serum) was stored at -80°C .

2.9. Measurement of Liver and Muscle Glycogen Content

The phenol–sulfuric acid method [22] was used according to a modified version [23]. About 0.5 g of tissue was disintegrated in a vial by adding 1.5 mL of 30% KOH solution and boiling for 40 min to form a homogenous solution. After cooling, 2 mL of 95% ethanol was used to precipitate the sugars in the homogenized tissues. This was performed at $2-8^{\circ}\text{C}$ for 30 min. The supernatant was recovered and removed by centrifuging at $1200\times g$ for 30 min at ambient temperature. The residue or sediment was washed twice with 95% ethanol and dissolved in 3 mL of distilled water. From the dissolved residue or glycogen standards (0.005–0.1 mg/200 μL), 200 μL was transferred into glass vials. Then, 1 mL of phenol (5% solution) and 5 mL of H_2SO_4 (98% solution) were added, successively. The vials were gently agitated to mix them. The absorbance of aliquots (250 μL) from each vial was measured at 490 nm (SpectraMax iD3 multi-mode microplate reader; Molecular Devices, San Jose, CA, USA). From the glycogen standard curve ($y = 0.021x - 0.0165$; $R^2 = 0.9984$), the glycogen content (mg/g tissue) of the tissues was calculated as follows:

$$\text{Tissue glycogen content (mg/g tissue)} = \frac{C \times D}{M}$$

where “C” is the extrapolated concentration (mg/200 μL); “D” is the dilution factor = 31, which is the dilution of 200 μL of the dissolved residue in 6.2 mL of assay volume; and “M” = 0.5 g, which is the mass (g) of tissue used in the assay.

2.10. Measurement of Serum Insulin

Serum insulin was measured using an enzyme-linked immunosorbent assay (ELISA) method. A Rat/Mouse Insulin ELISA Kit (Merck, Johannesburg, South Africa; product number: EZRMI) was used to perform the assay and the serum insulin concentration was expressed in ng/mL.

2.11. Tissue Homogenization and Protein Estimation

The tissues were thawed and homogenized using a method reported previously [23]. About 100 mg of the different tissues (liver, pancreas, and muscle) was homogenized in 1 mL of ice-cold buffer (sodium phosphate buffer containing 0.5% *v/v* Triton X-100 and 1 mM EDTA, pH 7.5) using a D1000 handheld homogenizer (Merck, Johannesburg, South Africa). The supernatant of the tissue homogenates was recovered at $10,400\times g$ for 10 min at 4°C . The protein concentration in the supernatant was measured. The protein concentration (mg/mL) was extrapolated from a bovine serum albumin (BSA) standard curve ($y = 0.7059x + 0.1982$; $R^2 = 0.9992$) using the bicinchoninic acid (BCA) method.

2.12. Measurement of Muscle Tissue Hexokinase Activity

The activity of hexokinase in the muscle tissue homogenate was measured at a protein concentration of 5 mg/mL according to the protocols described in a Hexokinase Colorimetric Assay Kit (Merck, Johannesburg, South Africa; catalogue number, MAK091). Hexokinase catalyzes the phosphorylation of glucose to glucose-6 phosphate. Glucose-6 phosphate is oxidized by glucose-6 phosphate dehydrogenase, during which the oxidized form of nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH. This assay estimates hexokinase activity by measuring the NADH-mediated colorimetric reduction of a chromogenic probe over 20 min. NADH production was quantified from an NADH standard curve ($y = 0.111x - 0.0147$; $R^2 = 0.998$) of NADH amounts (2.5, 5, 7.5, 10, and 12.5 nmol/reaction mixture) versus the corresponding absorbances at 450 nm. One unit

of hexokinase represents the enzyme activity that will generate 1.0 μmol of NADH from NAD^+ per minute at pH 8.0 under room temperature. The specific activity (nmol/min/mg protein) was calculated to the protein concentration of the tissue homogenates as follows:

$$\text{Specific activity (nmol/min/mg protein)} = \frac{A \times DF}{RT \times SV \times PC}$$

where “A” = amount (nmole) of NADH generated during reaction time; “DF” = dilution factor of sample in reaction volume; “RT” = reaction time (min); “SV” = sample volume (mL); and “PC” = sample protein concentration (mg/mL).

2.13. Measurement of Muscle Tissue Akt Phosphorylation

The pan-Akt and phosphor-Akt levels in the muscle tissue supernatant were estimated by a colorimetric ELISA method using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (Merck, Johannesburg, South Africa; catalogue number, RAB0012) and presented as the spectrometric optical densities (OD). To estimate the levels of Akt phosphorylation, the ratio between the ODs of phospho-Akt and pan-Akt was computed.

2.14. Measurement of Lipid Peroxidation

The protocol reported in a previous study [24] was slightly modified to perform this assay. Lipid peroxidation was estimated in the serum and tissue homogenates (liver and pancreas). In a vial, the tissue homogenate or malondialdehyde (MDA) standards (100 μL), 0.25% thiobarbituric acid solution (500 μL), 20% acetic acid solution (200 μL), and distilled water (200 μL) were successively added and mixed. The MDA standards were 7.5, 15, 22.5, 30, 45, and 60 μM solutions. The vials were placed in a boiling water bath for 50 min and the absorbance of aliquots (250 μL) from each vial was measured at 532 nm. Lipid peroxidation, determined as the concentration of thiobarbituric acid reactive substances (TBARS), was extrapolated from the MDA standard curve ($y = 0.0083x + 0.0062$; $R^2 = 0.999$).

2.15. Measurement of Superoxide Dismutase Activity

Superoxide dismutase activity (SOD) was measured in the serum and tissue homogenates (liver and pancreas) using a SOD Assay Kit (product number, 19160; Merck, Johannesburg, South Africa). SOD catalyzes the reduction of superoxide ions. Thus, the activity of SOD in the samples was determined by measuring the inhibitory action on superoxide ion-mediated colorimetric (450 nm) reduction of a water-soluble tetrazolium salt [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] as follows:

$$\text{Inhibition (\%)} = \frac{OD_C - OD_S}{OD_C} \times 100$$

where “OD_C” is the optical density for colorimetric (450 nm) reduction by the control (without enzyme), while “OD_S” is the optical density for colorimetric (450 nm) reduction by the samples.

The specific activity (nmol/min/mg protein) was calculated with respect to the protein concentration of the tissue homogenates as follows:

$$\text{Specific activity (\% inhibition/mg protein)} = \frac{I \times DF}{SV \times PC}$$

where “I” = percentage inhibition (%) by enzyme on superoxide ion-mediated colorimetric reduction of a water-soluble tetrazolium salt; “DF” = dilution factor of sample in reaction volume; “SV” = sample volume (mL); and “PC” = sample protein concentration (mg/mL).

2.16. Measurement of Catalase Activity

Catalase activity was measured in the serum and tissue homogenates (liver and pancreas) using a Catalase (CAT) Activity Assay Kit (Catalogue number, E-BC-K031-S;

Biocom Africa, Johannesburg, South Africa). Catalase catalyzes the decomposition of H_2O_2 , a reaction that can be chromogenically (405 nm) stopped by ammonium molybdate. The activity of catalase in the samples was estimated by measuring the residual H_2O_2 after 1 min of enzyme-substrate reaction. The residual H_2O_2 was extrapolated from a H_2O_2 standard curve ($y = 0.0307x - 0.0002$; $R^2 = 0.9998$) of H_2O_2 amounts (0, 0.5, 1, 2, 3, 4, and 5 μmol) versus the corresponding absorbances at 405 nm. The specific activity (U/mg protein) was calculated with respect to the protein concentration of the tissue homogenates as shown below. One unit of catalase will decompose 1.0 micromole of H_2O_2 per minute at pH 7.0 under room temperature at a substrate concentration of 50 mM H_2O_2 .

$$\text{Specific activity (U/mg protein)} = \frac{A \times DF}{RT \times SV \times PC}$$

where “A” = amount (μmole) of H_2O_2 that was used up during reaction time; “DF” = dilution factor of sample in reaction volume; “RT” = reaction time (min); “SV” = sample volume (mL); and “PC” = sample protein concentration (mg/mL).

2.17. Histopathological Examination of Pancreatic Tissues

The fixed pancreatic tissues were dehydrated by successive changes in concentrations (50%, 70%, 90%, and 100%, respectively) of ethanol. Ethanol was removed from the tissue by repeated immersions in xylene before being embedding in paraffin according to the standard protocol. The embedded tissues were cut into about 4 μm sections and mounted on slides. Hematoxylin and eosin were used to stain tissue samples to highlight important features of the tissue samples. The DPX mounting media was applied before placing a cover slip. The slides were viewed with a Leica slide scanner.

2.18. Data and Statistics Presentation

The data of this study were presented as the average of the replicate data ($n = \text{six}$ animals) along with the standard deviation ($\pm$). For each parameter, statistical analysis was conducted to compare the data mean of the different groups. This was performed using the IBM SPSS Statistics software (version 29). The one-way ANOVA and Tukey post hoc were used for computing the multiple comparisons of the data averages and statistical significance was set at $p < 0.05$.

3. Results and Discussion

The antidiabetic and antioxidant potential of litchi peel has been documented [9,12,14–16]. However, study trends show that the hydroalcoholic extracts have mostly been studied, while the ethyl acetate extract, which has been shown to have promising antidiabetic, phenolic, and flavonoid profiles [18], remains under-explored. The present study demonstrates the rich flavonoid profile of litchi peel ethyl acetate extract, as well as its ameliorative effects on T2D-related pathologies in a fructose/STZ model of diabetic rats.

3.1. Phytochemical Profile of Litchi Peel Ethyl Acetate Extracts

The hydroalcoholic extracts of litchi peel have been extensively studied. Documented evidence has shown that the hydroalcoholic extracts of litchi peel are rich in flavonoids including catechins, proanthocyanidins, and procyanidins, which are believed to be key bioactive flavonoids influencing the antioxidant, antidiabetic, anti-hypertensive, and anti-inflammatory potentials of the hydroalcoholic peel extracts [11,12,16,25]. On the other hand, a previous qualitative assessment [18] has suggested that the ethyl acetate extract of litchi peel may also be as rich in flavonoids as the hydroalcoholic extracts. However, specialized spectrometric profiling is still needed to confirm this. In our study, LC-MS analysis showed the notable presence of flavonoids such as epicatechin, cinnamtannin B2, procyanidin B5, and proanthocyanidin A2 in the ethyl acetate extract of litchi peel (Table 1, Figure 1). The presence of these flavonoids suggests that the ethyl acetate extract may also

potentiate antioxidant and antidiabetic effects, which have been investigated and reported in this study.

Table 1. LC-MS data of litchi peel ethyl acetate extract.

Identified Compounds	Ontology	ART (min)	AMz	Total Score	Peak Height	Conc. (mg/kg)
Aralidioside	Iridoid O-glycosides	5.648	447.11478	6.9378	12,228	502
Cinnamtannin B2	Flavonoids	5.923	575.11932	7.0967	17,074	701
Procyanidin B5	Flavonoids	6.635	577.13574	8.5544	6652	273
Epicatechin	Flavonoids	6.952	289.07196	8.1009	39,675	1630
Epicatechin-(4beta->8)-epicatechin-(2beta->7,4beta->8)-catechin	Flavonoids	7.168	863.18451	7.0737	286,371	11,763
Aesculitannin G	Flavonoids	7.917	574.11267	5.7575	90,599	3721
Proanthocyanidin A2	Flavonoids	8.306	575.12164	7.9145	110,862	4554
Quercetin 3-galactoside	Flavonoid-3-O-glycosides	8.447	463.08835	6.7863	10,707	440
epi-12-Palmatoside G	Naphthopyranone glycosides	8.671	491.19287	5.8164	11,744	482
Astragalin 7-rhamnoside	Flavonoid-7-O-glycosides	8.82	593.1521	7.0845	16,335	671

"ART", average retention time; "AMz", average mass-to-charge ratio; "Conc.", concentration.



Figure 1. LC-MS chromatogram of litchi peel ethyl acetate extract.

3.2. Glycemic Control and Antioxidant Potential of Litchi Peel Ethyl Acetate

Persistent hyperglycemia in a diabetic state causes excessive urinary excretion of glucose, which is responsible for some of the classical symptoms of diabetes, including weight loss, excessive food consumption (polyphagia), and excessive fluid consumption (polydipsia) [26]. In the present study, diabetic rats significantly ($p < 0.05$) lost weight and consumed more food and water compared to their nondiabetic counterparts (Table 2), which is influenced by their higher ($p < 0.05$) blood glucose levels (Figure 2). Treatment with the peel extracts (LDPE and HDPE) and metformin did not significantly improve the diabetic weight loss or fluid intake. The HDPE, however, had an appreciable positive effect on diabetic weight loss and fluid intake particularly in the fourth week of intervention. In fact, although not normalized, the HDPE significantly ($p < 0.05$) reduced diabetic polyphagia

(Table 2), suggesting a notable ameliorative effect of the litchi peel ethyl acetate extract, which can be further investigated long-term.

Table 2. Food intake, water intake, and weekly body weight.

Groups	Body Weight (g)						Food Intake (g/rat/d)	Water Intake (mL/rat/d)
	Week 0	Week 1	Week 2	Week 3	Week 3	Week 5		
NC	281 ± 9.97	307 ± 15.3 ^b	323 ± 17.7 ^b	324 ± 18.8 ^b	320 ± 19.5 ^b	328 ± 20.4 ^b	23.0 ± 1.25 ^c	47.7 ± 8.52 ^b
DBC	294 ± 16.1	279 ± 24.2 ^a	271 ± 25.9 ^a	256 ± 23.9 ^a	252 ± 28.0 ^a	245 ± 20.8 ^a	46.3 ± 2.86 ^a	160 ± 30.8 ^a
LDPE	291 ± 23.6	279 ± 18.4 ^a	279 ± 26.3 ^a	268 ± 23.5 ^a	257 ± 25.3 ^a	254 ± 32.0 ^a	37.6 ± 8.13 ^b	159 ± 26.6 ^a
HDPE	306 ± 11.2	289 ± 18.6 ^a	282 ± 21.9 ^a	273 ± 28.3 ^a	265 ± 26.5 ^a	265 ± 28.6 ^a	35.2 ± 1.19 ^b	136 ± 3.61 ^a
MET	302 ± 17.3	285 ± 20.3 ^a	268 ± 23.6 ^a	261 ± 24.8 ^a	252 ± 25.8 ^a	259 ± 29.4 ^a	40.9 ± 5.66 ^{ab}	152 ± 5.87 ^a

“NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. Week 0 is the STZ injection; Week 1 is the diabetes confirmation; Week 2 to Week 5 is the intervention period. For each parameter, a statistical comparison was conducted among the groups. A significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

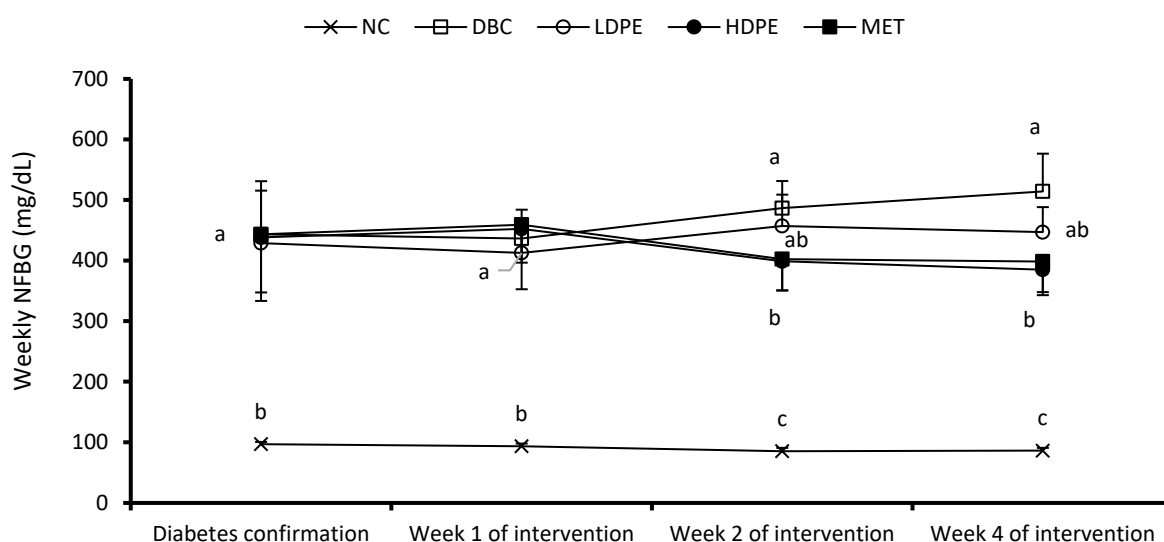


Figure 2. Weekly non-fasting blood glucose (NFBG). “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each period, a statistical comparison was conducted among the groups. A significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

The weekly NFBG data showed that the peel extract dose-dependently reduced NFBG over the treatment period (Figure 2). Notably, the HDPE caused a significant ($p < 0.05$) reduction in the second and fourth week of the intervention period, which was statistically comparable to that of metformin treatment, suggesting the blood glucose lowering potential of the peel’s ethyl acetate extract. Furthermore, the extract also exhibited a dose-dependent glucose tolerance effect (Figure 3a), with the effect of the HDPE being significant ($p < 0.05$) relative to the untreated diabetic animals (Figure 3b).

While the blood glucose-lowering effect of litchi peel ethyl acetate extract has been previously documented, the effect was demonstrated in alloxan-induced diabetic rats [18]. The alloxan-induced and STZ-induced diabetic models are characterized by oxidative stress-mediated pancreatic β -cell damage and impaired insulin secretion [27], which suggests that the previously documented blood glucose lowering effect of litchi peel ethyl acetate extract [18] may be influenced by an improvement in pancreatic β -cell damage, among other possible mechanisms. This is because supporting antioxidant data from the study by Chauhan et al. [18] showed that the ethyl acetate extract improved the pancreatic antioxidant status of the alloxan-induced diabetic rats.

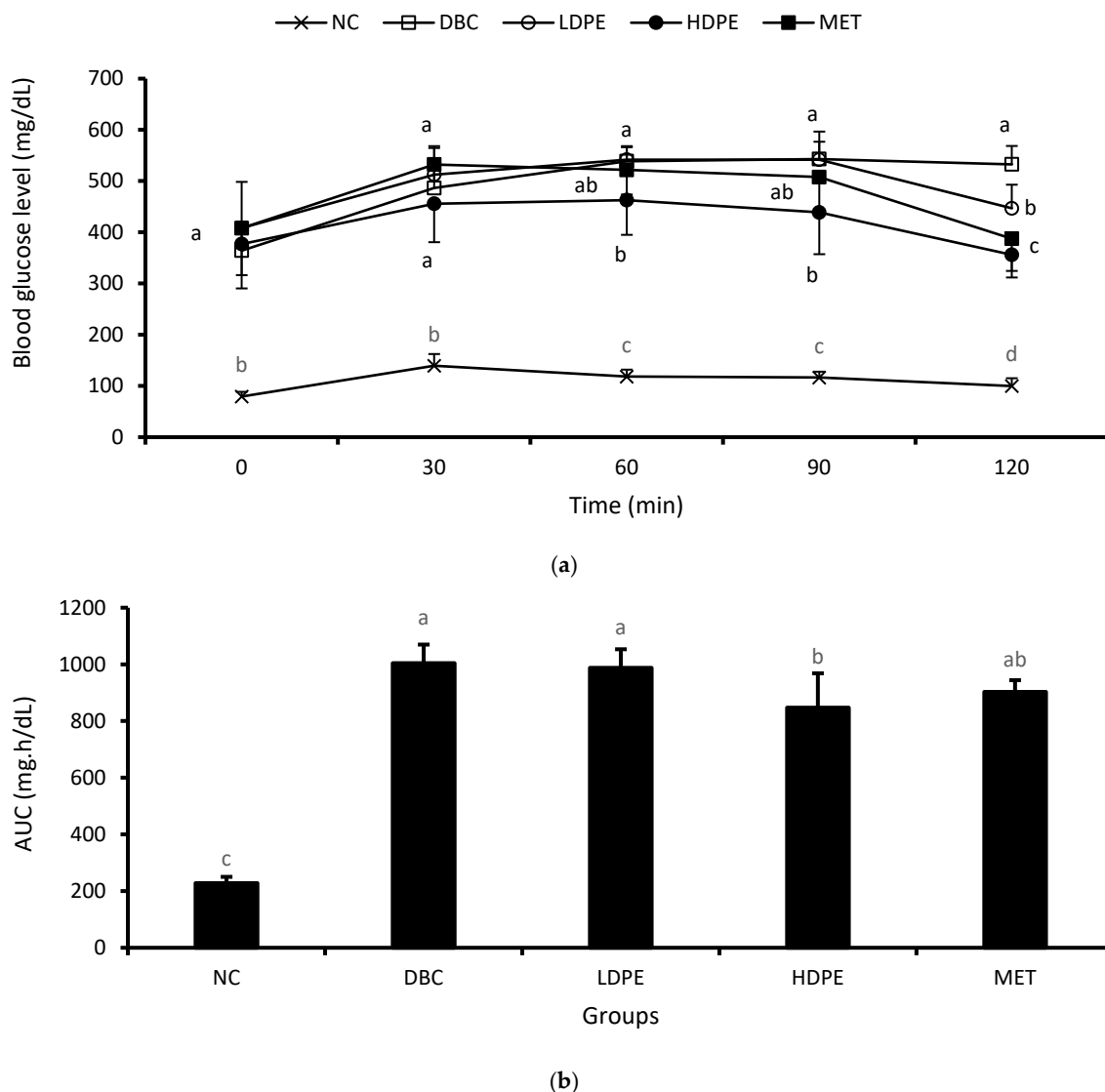


Figure 3. (a) Oral glucose tolerance data and (b) area under the curve. “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each period or parameter, a statistical comparison was performed among the groups. A significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

Histology data comparing the pancreas of the normal rats (NC) with that of the untreated (DBC) and treated (LDPE, HDPE, and MET) diabetic rats further reiterate the possible ameliorative effect of litchi peel ethyl acetate extract on chemical (STZ)-induced pancreatic damage (Figure 4). The pancreatic histology of the normal rats (NC) showed that the exocrine pancreatic acinar cells appear normal and active. The available endocrine islets consisted of normal cells. The distribution pattern and size of the endocrine islets of Langerhans are considered normal with no sign of cellular or exudate accumulation (Figure 4).

The pancreatic histology of the untreated diabetic rats (DBC) showed features consistent with multifocal and mild pancreatic endocrine islet cell degeneration, which was caused by the low-dose (40 mg/kg bw) STZ treatment during induction [27]. Typically, there were mild degenerative changes consisting of cytoplasmic vacuolization with mild swelling, mild lesions, and possible atrophy within cells of the pancreatic islet (Figure 4). Similarly, the pancreatic histology of the rats treated with peel extract and metformin (MET) showed features consistent with multifocal and mild pancreatic endocrine islet cell degeneration. However, the degeneration in the pancreatic islets of rats in the HDPE group

was minimal compared to that of the animals in the other groups (Figure 4), suggesting a somewhat ameliorated effect of the HDPE on STZ-induced pancreatic damage. This ameliorative effect further reflects the ability of the HDPE to significantly ($p < 0.05$) improve insulin secretion in diabetic rats as shown by the significantly ($p < 0.05$) higher serum insulin level compared to the untreated diabetic rats (Table 3).

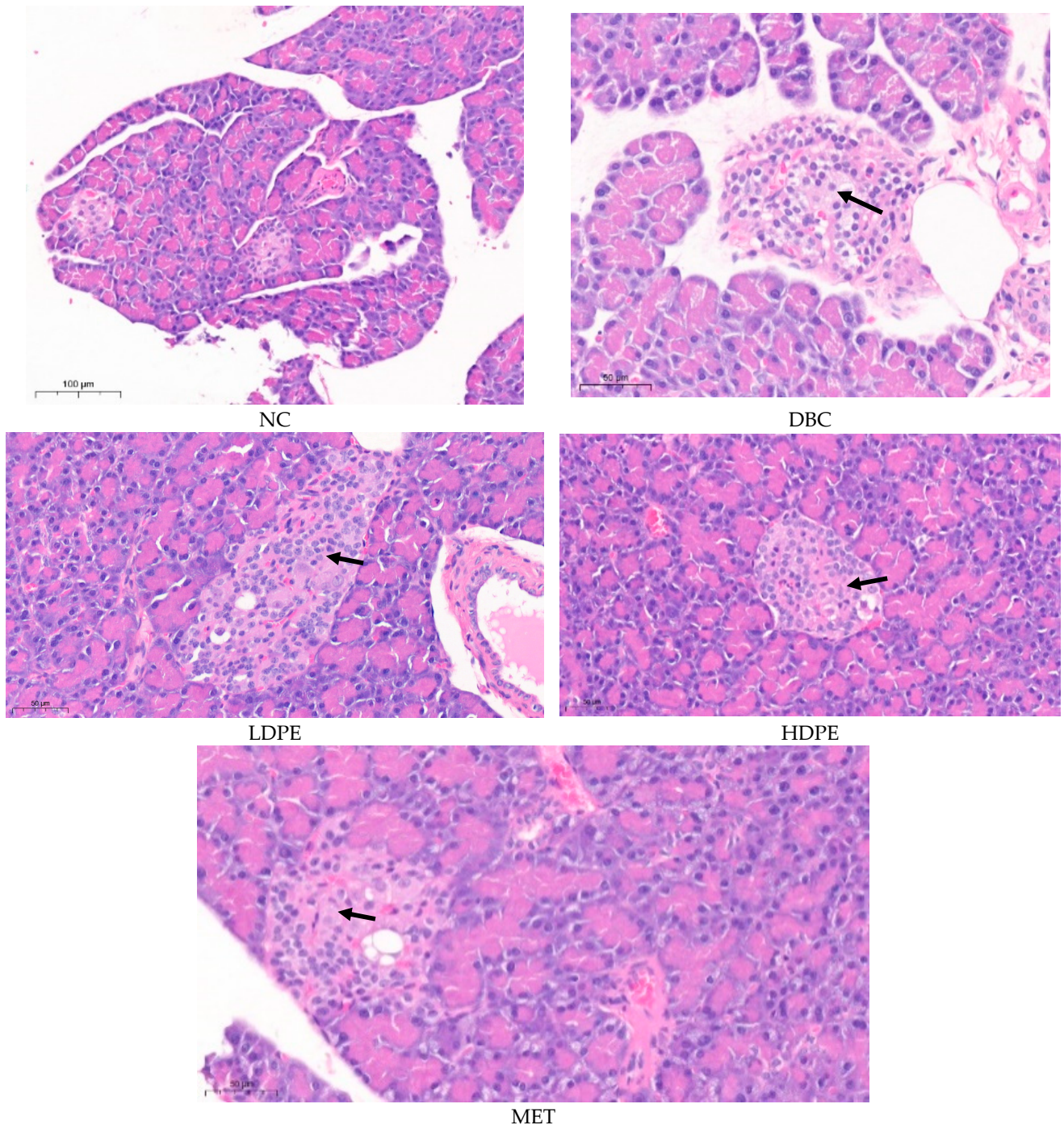


Figure 4. Histological images of the pancreatic tissues from rats. “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin.

Table 3. Serum insulin, tissue glycogen content, and muscle tissue hexokinase activity.

Groups	Serum Insulin Level (μ IU/mL)	Glycogen Content (mg/g tissue)		Muscle Hexokinase Activity (nmol/min/mg protein)
		Liver	Muscle	
NC	2.43 $\pm$ 0.31 ^b	17.3 $\pm$ 3.56 ^c	1.35 $\pm$ 0.23 ^b	19.0 $\pm$ 5.33 ^b
DBC	1.16 $\pm$ 0.36 ^a	9.61 $\pm$ 1.19 ^a	0.88 $\pm$ 0.08 ^a	10.2 $\pm$ 1.68 ^a
LDPE	1.50 $\pm$ 0.16 ^a	12.4 $\pm$ 4.65 ^{ab}	0.95 $\pm$ 0.19 ^{ab}	11.8 $\pm$ 3.58 ^a
HDPE	2.17 $\pm$ 0.51 ^b	14.6 $\pm$ 2.40 ^{abc}	1.10 $\pm$ 0.20 ^{ab}	15.3 $\pm$ 2.00 ^{ab}
MET	1.12 $\pm$ 0.11 ^a	16.2 $\pm$ 4.68 ^c	1.30 $\pm$ 0.15 ^b	16.5 $\pm$ 5.07 ^{ab}

“NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each parameter, a statistical comparison was conducted among the groups. A significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

It is plausible that the ameliorative effect of the HDPE on STZ-induced pancreatic damage may be linked to their pancreatic antioxidant capacities. The data of this study showed that the HDPE treatment significantly ($p < 0.05$) reduced pancreatic lipid peroxidation and concomitantly increased ($p < 0.05$) systemic and pancreatic catalase and SOD activities (Table 4).

Table 4. Lipid peroxidation and antioxidant enzyme activity in the serum and tissue of rats.

Groups	TBARS Level (μ M MDS Equivalent)			SOD Activity (% Inhibition rate/mg Protein)			Catalase Activity (μ mol/min/mg Protein)		
	Serum	Liver	Pancreas	Serum	Liver	Pancreas	Serum	Liver	Pancreas
NC	140 $\pm$ 28.0 ^c	78.0 $\pm$ 16.9 ^c	123 $\pm$ 37.3 ^c	90.1 $\pm$ 6.90 ^b	79.4 $\pm$ 5.91 ^b	72.2 $\pm$ 8.19 ^c	45.8 $\pm$ 7.24 ^b	126 $\pm$ 13.8	47.3 $\pm$ 4.00 ^d
DBC	564 $\pm$ 143 ^a	234 $\pm$ 23.0 ^a	294 $\pm$ 70.7 ^a	66.3 $\pm$ 4.09 ^a	66.4 $\pm$ 6.28 ^a	44.4 $\pm$ 3.89 ^a	27.3 $\pm$ 5.77 ^a	112 $\pm$ 24.4	15.2 $\pm$ 4.67 ^a
LDPE	419 $\pm$ 42.4 ^{ab}	209 $\pm$ 62.4 ^{ab}	222 $\pm$ 53.6 ^{ab}	84.0 $\pm$ 8.71 ^b	69.1 $\pm$ 1.24 ^a	48.2 $\pm$ 3.38 ^a	33.3 $\pm$ 6.67 ^a	123 $\pm$ 15.7	26.0 $\pm$ 2.32 ^b
HDPE	305 $\pm$ 62.1 ^a	146 $\pm$ 22.2 ^b	210 $\pm$ 20.2 ^b	85.8 $\pm$ 5.95 ^b	74.7 $\pm$ 4.25 ^{ab}	57.5 $\pm$ 2.00 ^b	35.4 $\pm$ 3.08 ^{ab}	131 $\pm$ 16.6	38.2 $\pm$ 3.85 ^c
MET	411 $\pm$ 117 ^b	217 $\pm$ 71.5 ^{ab}	257 $\pm$ 37.9 ^{ab}	70.5 $\pm$ 6.59 ^a	68.3 $\pm$ 7.80 ^a	47.4 $\pm$ 4.46 ^a	30.7 $\pm$ 9.03 ^a	116 $\pm$ 8.42	22.9 $\pm$ 8.35 ^{ab}

“NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each parameter, a statistical comparison was performed among the groups. A significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

Catalase and SOD are important antioxidant enzymes. SOD catalyzes the reduction of deleterious superoxide ions to hydrogen peroxide, which is reduced to water by the catalytic action of catalase [28]. Hence, the significantly higher pancreatic catalase and SOD activity of rats in the HDPE group, relative to the untreated diabetic rats, suggests that the HDPE-ameliorated STZ-induced ROS damage in the pancreatic tissue by mopping deleterious ROS. The potent in vivo antioxidant activity of the HDPE may be linked to the notable presence of bioactive flavonoids including catechins, proanthocyanidins, and procyanidins (Table 1 and Figure 1). Moreover, the hydroxyl radical and superoxide anion scavenging activity of proanthocyanidin B4, proanthocyanidin B2, and epicatechin isolated from litchi peel has been reported [11], while the modulatory effect of catechins and proanthocyanidins in vivo antioxidant status and antioxidant enzymes activity have been documented [29]. Furthermore, the key bioactive flavonoids present in the peel extracts may also influence its modulatory effect on insulin secretion. This is because a study has shown that epicatechin, a major flavonoid in the peel extract (Table 1 and Figure 1), modulates glucose-stimulated insulin secretion in INS-1 cells [30,31].

Other than the improvement in pancreatic antioxidant capacity, histology, and insulin secretion, our study further suggests that there are other possible mechanisms by which the litchi peel ethyl acetate extract improves glycemic control and ameliorates diabetic pathologies, which could be linked to the improvement in insulin action and glucose metabolism. In this study, we used fructose and low dose (40 mg/kg bw) STZ to induce diabetes, resulting in a model characterized by pancreatic β -cell damage and insulin resistance [20]. Moreover, the association between fructose consumption and insulin resistance in peripheral tissues, including the liver and muscle, has been documented [32]. Supporting data from our study showed that diabetes induction significantly ($p < 0.05$) reduced liver and muscle glycogen content (Table 3). Hepatic and muscle glycogen storage is one of the

insulin-mediated mechanisms for glycemic control and glucose homeostasis [33], suggesting that diabetes induction causes impaired insulin action or signaling. However, the litchi peel extract caused a dose-dependent increase in liver and muscle glycogen content in the diabetic rats. Although the effect of the litchi peel extract was not significant ($p > 0.05$), the effect of high-dose treatment (HDPE) was comparable to that of metformin treatment (Table 3), suggesting that litchi peel ethyl acetate extract potentiates glycemic control by promoting glycogen storage.

Furthermore, diabetes induction significantly ($p < 0.05$) reduced muscle hexokinase activity (Table 3). Hexokinase is a glycolytic enzyme that initiates the cellular utilization of glucose taken up into cells of peripheral tissues [34]. Glucose uptake and utilization are key insulin-mediated mechanisms for glucose homeostasis and glycemic control [33,34], suggesting that diabetes induction suppressed insulin-mediated glucose uptake and utilization in the muscle. Additional molecular investigation showed that diabetes induction significantly ($p < 0.05$) reduced muscle Akt phosphorylation (Figure 5), a key molecular step in the signaling of cell glucose uptake by insulin [35], buttressing the suppressive impact of diabetes induction on insulin-mediated glucose uptake and utilization in the muscle.

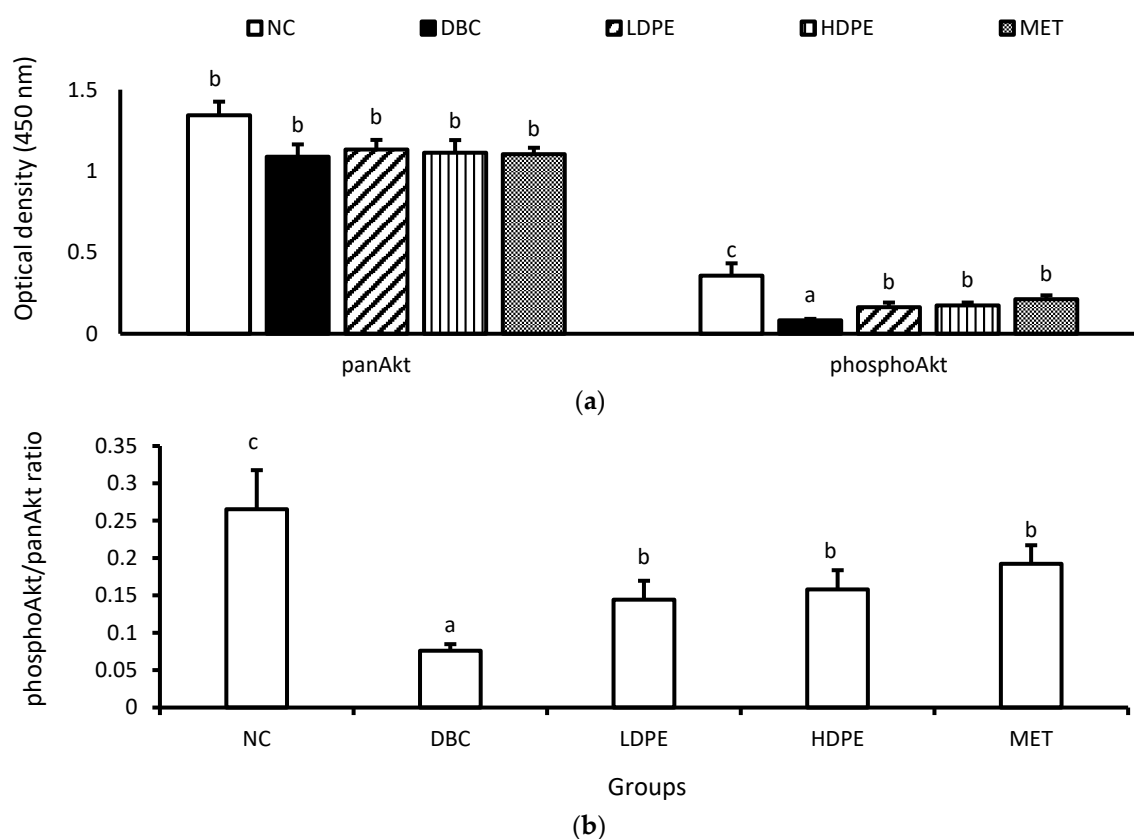


Figure 5. Muscle tissue (a) panAkt and phosphoAkt levels and (b) phosphoAkt/panAkt ratio of rats. “NC”, normal control; “DBC”, diabetic control; “LDPE”, low-dose peel extract; “HDPE”, high-dose peel extract; “MET”, metformin. For each parameter, statistical comparison was conducted among the groups. A significant difference ($p < 0.05$) exists between any two groups when there are no similar letters.

However, treatment with the peel extract, especially the high dose (HDPE), notably improved muscle hexokinase activity and Akt phosphorylation (Figure 5). Its modulatory effect on Akt phosphorylation was significant ($p < 0.05$) and comparable to that of metformin treatment, suggesting that the bioactive phytoconstituents in litchi peel ethyl acetate extract exerts a glycemic control effect via modulating cellular insulin signaling, glucose uptake, and utilization. Moreover, documented evidence has reported that epicatechin potentiates

glycemic control by modulating the IRS-1/PI3K/Akt signal transduction pathways, thus promoting glucose transporter type 4 (GLUT-4)-mediated cellular glucose uptake [29,31].

The comparative glycemic control potential of HDPE relative to metformin may be influenced by the observed multi-mode potential of HDPE. This includes improving pancreatic histology and antioxidant status, insulin secretion and signaling, tissue glycogen production, and cellular glucose uptake and utilization. While metformin may be a known standard drug, it may not be able to ameliorate some of the STZ-induced pathologies in the diabetic animals of this study. Documented evidence has shown that metformin could improve hepatic glycogen levels by suppressing glucagon action and gluconeogenesis [36]. It could also improve insulin signaling, as well as glucose uptake and utilization, thus improving glycemic control [36]. These modes of action are consistent with the glycogen content, Akt phosphorylation, and hexokinase activity data of the present study. However, metformin is not able to affect the STZ-induced pathologies (pancreatic oxidative stress, β -cell damage, and reduced insulin production) in diabetic rats because these are outside its pharmacological mechanisms. This could be the possible reason why metformin could not normalize the blood glucose levels, or glycemic control effect of the diabetic rat model used in the present study.

4. Conclusions

In the present study, the antidiabetic and antioxidant potential of litchi peel ethyl acetate extract was demonstrated in a fructose-STZ model of diabetic rats. The data of the study suggests the extract potentiated glycemic control by improving pancreatic histology and antioxidant status, improving insulin secretion, promoting glycogen storage, and modulating cellular glucose uptake and utilization. LC-MS analysis further revealed that flavonoids, including catechins and proanthocyanidins, were the major phytoconstituents of the extract, which, according to documented reports, are the possible influencing bioactive components of the extract. The ethyl acetate extract of litchi peel could be a source of bioactive flavonoids that can potentiate glycemic control in diabetes and mitigate oxidative-stress-related pathologies.

Author Contributions: G.O.I.: methodology, formal analysis, investigation, data curation, writing—original draft; S.S.M.: resources, supervision, writing—review and editing; C.I.C.: conceptualization, formal analysis, resources, supervision, funding acquisition, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: The study was funded by the postgraduate scholarship (reference: MND200429517886) and incentive funding for rated researchers (Grant No: 150945) of the National Research Foundation, South Africa.

Institutional Review Board Statement: The protocol reported in this study was approved by the Faculty Research and Innovation Committee of the Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein on 18 May 2021.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data of this study are available from the corresponding author on request.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. International Diabetes Federation. *IDF Diabetes Atlas*, 10th ed.; International Diabetes Federation: Brussels, Belgium, 2021. Available online: https://diabetesatlas.org/idfawp/resource-files/2021/07/IDF_Atlas_10th_Edition_2021.pdf (accessed on 2 January 2022).
2. Sanyaolu, A.; Marinkovic, A.; Prakash, S.; Williams, M.; Dixon, Y.; Okorie, C.; Orish, N.; Izurieta, R. Diabetes mellitus: An overview of the types, prevalence, comorbidity, complication, genetics, economic implication, and treatment. *World J. Meta-Anal.* **2023**, *11*, 134–143. [CrossRef]
3. Zhu, J.; Chen, M.; Pang, Y.; Li, S. Impact of lifestyle education for type 2 diabetes mellitus: Protocol for a randomized controlled trial. *J. Med.* **2021**, *100*, e24208. [CrossRef] [PubMed]

4. Galicia-Garcia, U.; Benito-Vicente, A.; Jebari, S.; Larrea-Sebal, A.; Siddiqi, H.; Uribe, K.; Ostolaza, H.; Martín, C. Pathophysiology of Type 2 Diabetes Mellitus. *Int. J. Mol. Sci.* **2020**, *21*, 6275. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Bhatti, S.; Sehrawat, A.; Mishra, J.; Sidhu, S.; Navik, U.; Khullar, N.; Kumar, S.; Bhatti, K.; Reddy, H. Oxidative stress in the pathophysiology of type 2 diabetes and related complications: Current therapeutics strategies and future perspectives. *Free Radic. Biol. Med.* **2022**, *184*, 114–134. [\[CrossRef\]](#)
6. Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Squadrito, F.; Altavilla, D.; Bitto, A. Oxidative Stress: Harms and Benefits for Human Health. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 8416763. [\[CrossRef\]](#)
7. Afam, J.; Silungwe, H.; Takalani, T.; Omolola, O.; Udeh, O.; Anyasi, A. Antioxidant-rich natural fruit and vegetable products and human health. *Int. J. Food Prop.* **2021**, *24*, 41–67.
8. Zhao, L.; Wang, K.; Wang, K.; Zhu, J.; Hu, Z. Nutrient components, health benefits, and safety of litchi (*Litchi chinensis* Sonn.): A review. *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 2139–2163. [\[CrossRef\]](#)
9. Chukwuma, I.; Izu, O.; Chukwuma, S.; Samson, S.; Makhafola, J.; Erukainure, L. A review on the medicinal potential, toxicology, and phytochemistry of litchi fruit peel and seed. *J. Food Biochem.* **2021**, *45*, e13997. [\[CrossRef\]](#)
10. Cano-Gómez, I.; Alonso-Castro, J.; Carranza-Alvarez, C.; Wong-Paz, E. Advancements in *Litchi chinensis* Peel Processing: A Scientific Review of Drying, Extraction, and Isolation of Its Bioactive Compounds. *Foods* **2024**, *13*, 1461. [\[CrossRef\]](#)
11. Zhao, M.; Yang, B.; Wang, J.; Li, B.; Jiang, Y. Identification of the major flavonoids from pericarp tissues of lychee fruit in relation to their antioxidant activities. *Food Chem.* **2006**, *98*, 539–544. [\[CrossRef\]](#)
12. Tanaka, R.; de Souza Mataruco, L.; Frota, G.; Coelho, S.; dos Santos Lima, M.; Pimentel, C.; Barão, E. Lychee peel extract obtained by ultrasound-assisted extraction: Bioactive compounds and functional properties. *Acta Sci. Technol.* **2024**, *46*, e66447. [\[CrossRef\]](#)
13. Derosa, G.; Maffioli, P. α -Glucosidase inhibitors and their use in clinical practice. *Arch. Med.* **2012**, *8*, 899–906. [\[CrossRef\]](#)
14. Rong, S.; Hu, X.; Zhao, S.; Zhao, Y.; Xiao, X.; Bao, W.; Liu, L. Procyanidins extracted from the litchi pericarp ameliorate atherosclerosis in ApoE knockout mice: Their effects on nitric oxide bioavailability and oxidative stress. *Food Funct.* **2017**, *8*, 4210–4216. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Queiroz, R.; Abreu, C.; Rocha, A.; Sousa, V.; Fráguas, M.; Braga, A.; César, P. Lychee (*Litchi chinensis* Sonn.) peel flour: Effects on hepatoprotection and dyslipidemia induced by a hypercholesterolemic diet. *An. Acad. Bras. Ciências* **2018**, *90*, 267–281. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Yang, Z.; Zhang, L.; Liu, J.; Li, D. Litchi Pericarp Extract Treats Type 2 Diabetes Mellitus by Regulating Oxidative Stress, Inflammatory Response, and Energy Metabolism. *Antioxidants* **2024**, *13*, 495. [\[CrossRef\]](#)
17. Prasad, N.; Chew, Y.; Khoo, E.; Kong, W.; Azlan, A.; Ismail, A. Antioxidant capacities of peel, pulp, and seed fractions of *Canarium odontophyllum* Miq. fruit. *J. Biomed. Biotechnol.* **2010**, *2010*, 871379. [\[CrossRef\]](#)
18. Chauhan, S.; Gupta, S.; Yasmin, S.; Saini, M. Antihyperglycemic and Antioxidant Potential of Plant Extract of *Litchi chinensis* and *Glycine max*. *Int. J. Nutr. Pharmacol. Neurol. Dis.* **2021**, *11*, 225–233. [\[CrossRef\]](#)
19. Yang, R.; Tang, X.; Zhou, Y.; Wu, Q.; Sun, Y. Effect of ethyl acetate extracts from litchi fruit on the Th1/Th2 cell balance in mice. *Mod. Food Sci. Technol.* **2013**, *29*, 1467–1470.
20. Wilson, D.; Islam, S. Fructose-fed streptozotocin-injected rat: An alternative model for type 2 diabetes. *Pharmacol. Rep.* **2012**, *64*, 129–139. [\[CrossRef\]](#)
21. Sakaguchi, K.; Takeda, K.; Maeda, M.; Ogawa, W.; Sato, T.; Okada, S.; Ohnishi, Y.; Nakajima, H.; Kashiwagi, A. Glucose area under the curve during oral glucose tolerance test as an index of glucose intolerance. *Diabetol. Int.* **2015**, *7*, 53–58. [\[CrossRef\]](#)
22. Lo, S.; Russel, C.; and Taylor, W. Determination of glycogen in small tissue samples. *J. Appl. Physiol.* **1970**, *28*, 234–236. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Matowane, R.; Mashele, S.; Makhafola, J.; Chukwuma, I. The ameliorative effect of zinc acetate with caffeic acid in the animal model of type 2 diabetes. *Biomed. Pharmacother.* **2023**, *163*, 114779. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Tshane, L.; Mashele, S.; Matowane, R.; Bonnet, L.; Makhafola, J.; Noreljaleel, M.; Swain, S.; Sekhoacha, M.; Chukwuma, I. Zinc(II) mineral increased the in vitro, cellular and ex vivo antihyperglycemic and antioxidative pharmacological profile of p-hydroxybenzoic acid upon complexation. *J. Food Biochem.* **2021**, *45*, e13609. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Li, J.; Jiang, Y. Litchi flavonoids: Isolation, identification and biological activity. *Molecules* **2007**, *12*, 745–758. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Nigro, N.; Grossmann, M.; Chiang, C.; Inder, J. Polyuria-polydipsia syndrome: A diagnostic challenge. *Intern. Med. J.* **2018**, *48*, 244–253. [\[CrossRef\]](#)
27. Lenzen, S. The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia* **2008**, *51*, 216–226. [\[CrossRef\]](#)
28. Lee, E.; Park, S. The Emerging Roles of Antioxidant Enzymes by Dietary Phytochemicals in Vascular Diseases. *Life* **2021**, *11*, 199. [\[CrossRef\]](#)
29. Patanè, T.; Putaggio, S.; Tellone, E.; Barreca, D.; Ficarra, S.; Maffei, C.; Calderaro, A.; Laganà, G. Catechins and Proanthocyanidins Involvement in Metabolic Syndrome. *Int. J. Mol. Sci.* **2023**, *24*, 9228. [\[CrossRef\]](#)
30. Yang, K.; Chan, C. Epicatechin potentiation of glucose-stimulated insulin secretion in INS-1 cells is not dependent on its antioxidant activity. *Acta Pharmacol. Sin.* **2018**, *39*, 893–902. [\[CrossRef\]](#)
31. Martín, M.Á.; Goya, L.; Ramos, S. Antidiabetic actions of cocoa flavanols. *Mol. Nutr. Food Res.* **2016**, *60*, 1756–1769. [\[CrossRef\]](#)
32. Taskinen, R.; Packard, J.; Borén, J. Dietary Fructose and the Metabolic Syndrome. *Nutrients* **2019**, *11*, 1987. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Röder, V.; Wu, B.; Liu, Y.; Han, W. Pancreatic regulation of glucose homeostasis. *Exp. Mol. Med.* **2016**, *48*, e219. [\[CrossRef\]](#) [\[PubMed\]](#)

34. Wasserman, H. Insulin, Muscle Glucose Uptake, and Hexokinase: Revisiting the Road Not Taken. *Physiology* **2022**, *37*, 115–127. [[CrossRef](#)] [[PubMed](#)]
35. Chang, L.; Chiang, H.; Saltiel, R. Insulin Signaling and the Regulation of Glucose Transport. *Mol. Med.* **2004**, *10*, 65–71. [[CrossRef](#)]
36. Rena, G.; Hardie, D.G.; Pearson, E.R. The mechanisms of action of metformin. *Diabetologia* **2017**, *60*, 1577–1585. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.



Certificate of Participation

This is to confirm that

Ms Gloria Izu

Has attended the 2023 Conference
of
The South African Society for Basic and Clinical Pharmacology
at Rhodes University
on the 24th - 27th August 2023

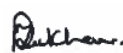
(accredited for 18 CPD Points)



“Pharmacology celebrating resilience: Current and Future strategies”



Prof Mamza Mothibe
Chairperson: 2023 Organising Committee



Ms Tanusha Dukhan
Secretary: 2023 Organising Committee

Animal Research Ethics

07-Feb-2023

Dear Dr Gloria Izu

Student Project Number: UFS-AED2022/0028/23

Project Title: Investigating the antidiabetic and antioxidative potential of peels from selected South African fruits

Department: External Affiliation

You are hereby kindly informed that, the UFS Animal Research Ethics Committee approved the above project.

Kindly take note of the following:

1.

A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.

Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.

Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely



Dr Walter Janse van Rensburg
Chair: Animal Research Ethics Committee